Research on Captive Broodstock Programs for Pacific Salmon

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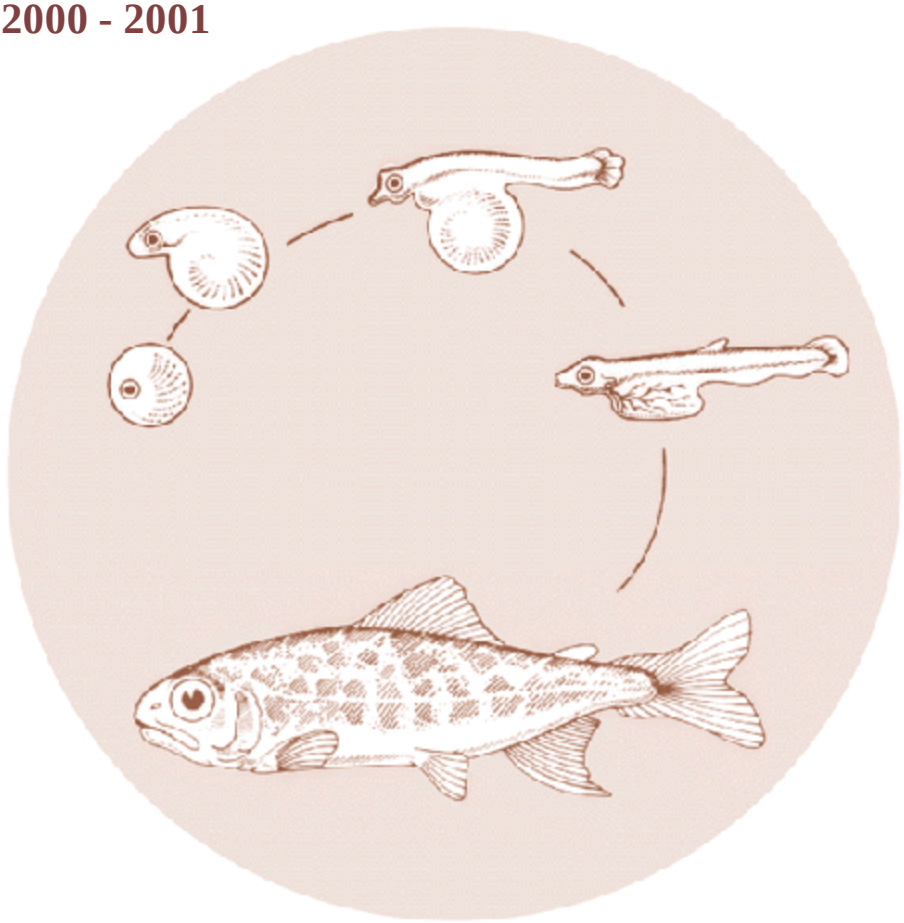

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RESEARCH ON CAPTIVE BROODSTOCK PROGRAMS FOR PACIFIC SALMON

ANNUAL REPORT
(PERFORMANCE PERIOD: 1 JUNE 2000 THROUGH 31 MAY 2001)

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EXECUTIVE SUMMARY

The efficacy of captive broodstock programs depends on high in-culture survival and the fitness of cultured salmon after release, either as adults or juveniles. Continuing captive broodstock research designed to improve technology is being conducted to cover all major life history stages of Pacific Salmon. The following are some of the salient results and accomplishments from the FY 2000 performance period:

- During the spring outmigration, wild Redfish Lake sockeye salmon had twice as much lipid as hatchery fish that were released into the lake the previous fall. However, sockeye salmon reared to the smolt stage in the hatchery contained more than twice as much whole body lipid as did wild fish, between 30 and 38% on a dry-weight basis.
- Treatment of maturing captively-reared spring chinook salmon with GnRHa affected several reproductive behaviors of females that are likely to improve their reproductive performance.
- A threshold size for initiation of sexual maturation in male spring chinook salmon appears to be between 15 and 30 g in early December.
- Reducing ration size for juvenile chinook salmon during winter months is a viable management strategy to reduce feed waste and pollution from hatcheries, providing that feed is provided in such a way as to minimize size differences within a tank or raceway.
- A genetic analysis of chinook salmon estimated the heritability of adult reproductive characteristics to determine how they would respond to various forms of selection.
- Study of captively reared coho salmon suggests initiation of maturation occurs in the fall, followed by a permissive period the following spring. Growth during the summer and early fall just prior to spawning was the strongest determinant of final body size, total ovary mass, and fecundity.
- Azithromycin is moderately more effective in reducing mortality due to BKD in captive broodstock salmon than erythromycin.

REPORT STRUCTURE

This report is organized by Task number as outlined in the FY 2000 Statement of Work
For each Task there is included either a Final Report or a Progress Report.

Final reports include data presentation, statistical analysis, and an interpretation (i.e., discussion) of the results for the performance period indicated. Final reports are not meant to imply that the research has been completed, only that the reporting for the noted performance period is complete. In many cases, further research on the same or similar topics may be necessary, and therefore continued in future years under this project. Final reports from previous performance periods are in Appendices at the end of the report.

Progress reports have been included for Tasks that extend beyond the performance period or for experiments in which data analysis has not been completed. Preliminary data have purposely been excluded from some progress reports to eliminate potential problems with misinterpretation of incomplete data sets and analyses. Final reports for these tasks will be completed according to the schedules described in the Work Statement.

PEER-REVIEWED PUBLICATIONS OF THE PROJECT

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TASK 1. DETERMINE THE EFFECTS OF CONSTANT AND VARIABLE DIETARY PROTEIN AND ENERGY INTAKES ON POST-RELEASE FITNESS OF JUVENILE CHINOOK SALMON

(PROGRESS REPORT: 1 JUNE 2000 THROUGH 31 MAY 2001)

by

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Introduction

It is not yet possible to define a feeding regimen for captively-reared stocks similar to their natural regimen that enhances the post-release fitness of juveniles and improves the reproductive performance of adults. In the natural environment, seasonal differences in food quality and quantity have profound effects on growth and 'wild' attributes, such as external coloration and fin quality. Formulating the right feeds for conservation fish held for long periods in captivity before release is more complicated than formulating diets for farm fish. Recent research in salmonid nutrition shows it is necessary to consider daily dietary protein intake and protein intake relative to total dietary energy level, rather than simply the levels of total dietary lipid.

The proposed approach is to formulate diets and develop a feeding regimen for captively-reared chinook salmon, from juveniles to mature adults, which mimic seasonal differences in food availability in the environment. In the second year, the work includes enhancing juvenile quality for post-release fitness and carrying experimental fish into the post-juvenile period.

Different diets and regimens will be evaluated by their effects on fitness for post-release survival, including growth, body conformation, and color. Specific tasks will investigate the effects of varying daily protein intake (g digestible protein/kg/d) and daily energy intake (kcals/kg/d) on protein and energy accretion in growing juveniles before release. Continuing work will investigate the effects of selected dietary treatments on age-at-maturity, and on adult breeding success in years 3 and 4.

The natural diets of juvenile salmonids in freshwater consist mainly of aquatic and terrestrial insects. Many published studies have documented the natural diet of juveniles, both in terms of prey and nutrient intake. The studies show conclusively that the proximate composition of natural diets is approximately 45% protein and 15 - 17% lipid. Although the natural diet of juvenile chinook salmon in freshwater consists of insects, with lipid levels ranging from 2 - 39% on a dry weight basis, the four main groups of insects consumed by juvenile chinook average 53% protein and 16.5% lipid. This is similar to the composition of freshwater copepods and small fishes. Wild juvenile chinook are distinctly different from hatchery-reared chinook in body fat deposits, particularly in the amount of fat in visceral stores, and in external coloration and fin quality. These differences are greater among stream-type juvenile chinook than among ocean-type.

The body conformation of chinook salmon reared in captive broodstock programs differs from that of wild fish, mainly in length to girth ratio. Captively-reared fish have a lower length to girth ratio, meaning that they tend to be shorter and fatter. This difference is presumably related to dietary energy intake and level of activity. Dietary energy intake is similar between wild and captively-reared fish when expressed in terms of proximate composition, or percentage of the diet as protein and lipid. However, dietary energy intake is a function of the proximate composition of the diet as well as feed intake. Wild fish obviously consume less food during certain periods of the year. Recent studies with juvenile chinook salmon (funded by BPA) have identified annual variations in levels of the metabolic hormone IGF-1 and growth hormone (W.W. Dickhoff, NMFS, personal communication). This provides a metabolic rationale for numerous observations made over 40 years concerning the higher level of whole body lipid in hatchery-reared fish compared with that in wild salmon. During periods of declining day length circulating levels of these hormones are low and protein synthesis rates in the body are reduced. Fish convert both dietary protein and lipid into stored body fat during these periods. In contrast, when day lengths increase, body metabolism patterns change, resulting in increases in protein synthesis rates and lypolysis, and thus lower percentage whole body fat levels. When feeding levels are adjusted to correspond with this pattern of anabolism and catabolism, survival to hatchery return increases.

Dietary requirements also change as fish grow and develop. It is well known that the scope for growth of Pacific salmon decreases with fish size. Thus, the protein needs of salmon, expressed as a percentage of dietary metabolic energy, decrease with fish size. Juvenile chinook salmon require between 1.3 - 1.6 g protein/kg/d to continue to grow. The amount of protein intake to support weight gain varies with fish size and dietary energy intake, but growth increases linearly in juvenile salmonids consuming between 6 - 18 g protein/kg/d.

Work Completed

In January 1999, chinook salmon (Clearwater River Strain, 1998 brood year) were obtained as first-feeding fry from the IDFG Clearwater Hatchery and transferred to the

fish culture laboratory of the Aquaculture Research Institute, University of Idaho. The fish were fed a commercial salmon starter diet (BioDiet) until they reached an average weight of 13 g on 3 December. The fish were then graded and counted into groups of 25. Each group was bulk-weighed and placed in one of 12 experimental rearing tanks (400 L), using a round-robin procedure, until each tank contained 150 fish. Dietary treatments were assigned randomly, with replicate groups receiving each dietary treatment. The tanks were supplied with temperature-controlled, recycled freshwater (9 - 11 ^{o}C), and the lighting was maintained under the photoperiod regime of the area.

The fish were fed the first group of experimental diets, which have been assayed by batch for proximate composition. All feeds contained the appropriate levels of protein and lipid. Feeding frequency was four times per day to begin, but decreased to two feedings per day by the end of the reporting period. Feeding was by hand 5 d/wk. Four dietary treatments were used in this trial:

- Diet #1. Constant diet, to provide 9 g protein/kg/d,
- Diet #2. Constant diet with variable feeding level, to provide 6 - 12 g protein/kg/d with proportional dietary energy intake,
- Diet #3. Variable diet adjusted to provide 6 - 12 g protein/kg/d at a constant dietary energy intake of 75 kcal/kg fish/d,
- Diet #4. high-protein, low-fat diet in which most of the dietary energy was supplied from protein. This diet supplied 12 g protein/kg/d at a total dietary energy intake, similar to #1 and #3.

Each diet was fed to three replicate tanks of fish. The feed formulation for the three diets is shown in Table 1, and the proximate composition of the diets in Table 2. The feed was produced at the Hagerman Fish Culture Experiment Station by compression-pelleting. The variable dietary treatments (#2 and #3) provided the fish with the lowest protein intake during late early winter months, and the highest protein intake during spring and early summer months. This was accomplished by producing different feeds for each seasonal period, and by adjusting feeding level to supply the desired protein and energy intake per fish per day. The constant dietary treatment (#1) was fed at 2% of the biomass in each tank per day during the first period, but this level of feeding was slightly more than the fish would consume, so all feeding levels were reduced to 1.5%. Feed amount was adjusted weekly for estimated growth and adjusted after the fish were weighed and counted based upon actual growth. Feed consumption was recorded daily to monitor feed intake and to permit feed efficiency ratios to be calculated for each growth period.

For the first 10 weeks the fish in each tank were bulk-weighed and counted every 2 weeks after a 2-day period of fasting (weekend). The average length was determined by measuring the fork-length of a sample of 50 fish from each tank. The average weight and length of fish in each replicate tank were then calculated, as was the variation in length among fish within tanks. The fish were weighed and counted monthly after the tenth week of the feeding trial to reduce handling stress.

An initial sample of 20 fish, followed at selected intervals by 12 fish from each dietary treatment group (three per replicate tank), were removed and used for proximate analyses. The analyses were conducted at Hagerman using standard procedures (AOAC 1990). The proximate composition data were used to calculate protein and energy retention at various stages of growth in the fish associated with dietary treatment and season. Data were transformed if necessary and statistically analyzed using a computer program (GraphPad Prism, version 2.01, GraphPad Software, Inc., San Diego, CA). Treatment effects were considered significant at $P<0.05$.

Chinook from the smolt feeding study were reared on the variable dietary protein and energy intake treatments from December 1999 through July 2000. The following values were calculated:

- Weight and length gain per period
- Feed intake per period
- Feed efficiency ratio (fish weight gain (g)/ feed fed per fish (g)
- Specific growth rate
- Protein retention (protein gain per period / protein fed), also referred to as nitrogen retention
- Energy reserves, expressed as total body lipid (both as a percentage and as an absolute amount, g lipid/g body weight
- Fin quality and body conformation, based upon dorsal fin index (DFI),and condition factor, respectively

Feeding periods were established based upon the hypothesis that winter dormancy in these fish extends during the months when stream temperatures and food availability is low, roughly from the beginning of the experimental period to the end of April (in Idaho). Thus, the feeding trial was divided into two periods reported herein, namely winter and spring. During the winter period, fish in dietary treatment group #2 were fed the same diet as fish in dietary treatment group #1, but at one-half the feeding level. Fish in dietary treatment group #3 were fed a low-protein diet during the winter, such that they consumed the same quantity of dietary energy as fish in dietary treatment group #1, and the same dietary protein as fish in dietary treatment group #2. When the spring period began, the feeding level of fish in dietary treatment group #2 was increased to be equivalent (on a percentage body weight basis) as fish in dietary treatment group #1. Fish in dietary treatment group #3 were fed a higher protein diet, still receiving the same dietary energy as fish in dietary treatment group #1. In other words, they consumed less protein per fish per day during the winter and more protein per fish per day during spring than fish in dietary treatment group #2.

The starting weight and length of the fish were 13 g and 107 mm, respectively, in all treatment groups, and at the end of the winter period, the average fish weight in dietary treatment group #1 was 34.5 g, compared with 29.6 g for fish in dietary treatment group #3 (Table 3). Average fish fork length followed the same pattern with respect to treatment effects as fish weight. Feed intake was roughly double for fish fed diets #1 and #4 compared with fish fed diets #2 and #3, and this resulted in large differences in feed efficiency ratios among the dietary treatment groups (Table 4). Protein retention was

much higher in fish in dietary treatment groups #2 and #3 compared with fish in groups #1 and #4, with fish fed diet #4, the highest protein intake, exhibiting the lowest protein retention value. No differences were noted in the percentage of crude protein in fish sampled at the end of the winter period despite large differences in protein intake, but large significant differences were found in percentage whole body lipid (Table 5), associated with both dietary energy intake and dietary lipid intake. Expressing whole body proximate composition on an absolute basis (g/g body weight) revealed differences associated with fish size and dietary treatment group, specifically mg protein or kcal energy intake/day/fish (Table 6).

Changing the dietary composition and/or feeding level during the spring period reduced the differences in feed intake, fork length, and weight gain among dietary treatment groups during this period (Table 7). Derived indices of performance, i.e., specific growth rate, feed efficiency ratio, and protein retention percentage were similar among dietary treatment groups compared with values calculated during the winter period (Table 8). Condition factor values were nearly identical between fish in dietary treatment #1 and dietary treatment #2, and identical between fish in dietary treatments #3 and #4 (Table 8), despite similar final fish weights between dietary treatment groups #1 and #4, and #2 and #3, respectively. Thus, dietary treatment influenced fish conformation, despite similar feed intake values between groups having dissimilar condition factors. Dorsal fin height was similar in all treatment groups at the end of the study period (Table 8). The proximate composition of the fish followed the same pattern observed in the winter period, with respect to the relative rankings among treatment groups (Table 9). However, percent lipid was lower in all dietary treatment groups except for group #2 between the winter and spring periods. Expressing proximate composition on an absolute basis revealed the same pattern of composition as that observed at the end of the winter period (Table 10), except that there was evidence of compensatory lipid (energy) deposition in fish in dietary treatment group #2 after restoration of the feeding level to that being received by dietary treatment group 1 (both groups received the same diet, but at different feeding levels during the winter period).

The results of this study confirm that the potential for actual growth during winter months is relatively low in juvenile chinook salmon and, further, that providing feed during this period at amounts recommended by feeding tables is wasteful, both in terms of low efficiency of feed utilization and nitrogen losses in hatchery discharge water (low protein retention equals high nitrogen loss). Feeding fish at half of the level of full fed fish during the winter period resulted in slightly smaller fish at the end of the winter period, but by the end of the spring period, during which time the feeding level was restored to recommended levels, the fish were close in average weight to full-fed fish. Further, weight and length gains between the two groups were virtually identical during the spring period, showing that there was no negative effect on fish growth associated with underfeeding during the winter period, an observation supported by the specific growth rates of the two dietary treatment groups. Moreover, the proximate composition of the fish was nearly identical at the end of the spring period (less than 0.5% difference in percent lipid content). Thus, reducing ration size during winter months is a viable

management strategy to reduce feed waste and pollution from hatcheries, providing that feed is provided in such a way as to minimize size differences within a tank or raceway.

Providing a constant dietary input of feed energy but varying the dietary input of protein with season yielded leaner fish at the end of both winter and spring feeding periods compared with simply reducing the feeding level of a conventional diet. Fish in dietary treatment #3 were nearly 2% lower in whole body lipid content than fish fed a conventional diet at full-feeding, despite the fact that in both dietary groups, energy intake was equivalent. Further, these fish had a more elongated body shape, as indicated by the lower condition factor value. Even more extreme body composition was achieved by feeding an extreme diet. The disadvantage of this extreme diet is that it is difficult to formulate and extremely difficult to manufacture. In addition, the extreme diet was found to yield the lowest protein retention value, and thus would be the least "environmentally-friendly" of the tested diets during the winter period of low protein accretion. Nevertheless, this extreme diet resulted in the leanest fish, and in fish having a condition factor value that indicated a favorable body length to girth ratio (more like wild-type fish).

External coloration: Fish from the dietary treatment groups were examined visually for differences in external coloration, but no differences were visible at the end of the spring period. All fish were silvery, with black coloration on the distal edges of fins consistent with typical salmon smolts. Thus no further color analysis was attempted.

Myosin expression: A new technique was developed to measure myosin expression levels in fish muscle. This technique relies on quantitative measures of copies of myosin RNA isolated from the muscle, and prepared using PCR and a fluorescence-labeled single-stranded DNA probe that hybridizes specifically to a region within the myosin mRNA of rainbow trout. It was possible to develop and validate this technique using rainbow trout, but it was not possible to apply the technique to the chinook salmon in this study due to delays in validating the technique. Because the technique requires fresh samples (RNA levels degrade quickly after death) samples could not be stored for later analysis. Nevertheless, the technique was tested on steelhead trout reared at the Hagerman National Fish Hatchery during fall, winter and spring (2000-2001) and significant changes in myosin expression were found over the period. Specifically expression levels declined from late fall to early winter and then returned to normal levels.

Table 1. Composition of experimental diets for chinook salmon captive broodstock studies in Task 2.

Ingredient	Diets #1 & #2 (%)[1]	Diet #3 (%)[1]	Diet #4 (%)[1]
Anchovy meal	54.00	30.00, 50.50, or 65.00	68.50
Blood meal	4.00	4.00	7.00
Wheat gluten	4.00	4.00	15.00
Wheat mids	16.00	12.50 to 19.50	2.27
Fish oil	13.00	6.00 to 13.00	0.00
Vitamin C	0.30	0.30	0.30
Choline	0.50	0.50	0.50
TM salt[2]	0.10	0.10	0.10
Vitamin premix[3]	3.00	3.00	0.30
Krill hydrolysate	5.00	5.00	0.50
Astaxanthin[4]			0.10

Footnotes:

[1] Calculated proximate composition of Diets #1 & #2 = 48% crude protein (45% digestible protein) and 17.5% crude lipid, expressed on an "as-is" basis (7% moisture). Diet #3 varies to keep energy intake constant while varying daily protein intake (g protein/kg fish/d). Diet #4 contained 65% crude protein and 5% crude lipid.

[2] Supplies the following per kg dry diet: Zn as $ZnSO_4.7\ H_2O$, 75 mg; Mn as $MnSO_4.H_2O$, 20 mg; Cu as $CuSO_4.5\ H_2O$, 1.54 mg; I as $KIO.C_2H_8N_2$ .2HI, 10 mg.

[3] Supplies the following per kg dry diet: thiamin mononitrate, 62 mg; riboflavin, 71 mg; niacin, 294 mg; calcium pantothenate, 153 mg; pyridoxine hydrochloride, 50 mg; folic acid, 22 mg; vitamin B12, 0.08 mg; d-biotin, 0.8 mg; myoinositol, 176 mg; retinol acetate, 8818 IU; vitamin D3, 588 mg; (α-tocopherol acetate, 670 mg; menadione sodium bisulfite complex, 37 mg.

[4] Carophyll Pink, Hoffman La-Roche, Basle, Switzerland.

Table 2. Protein and energy intake during feeding periods, expressed as g protein/kg fish/day for the experimental treatment groups.

Dietary treatment	Protein	Energy
1 (All seasons)	9	Constant
2 (Fall)	4.5	Half of diet #1
2 (Winter)	4.5	Half of diet #1
2 (Spring)	9	Same as diet #1
2 (Summer)	9	Same as diet #1
3 (Fall)	9	Same as diet #1
3 (Winter)	3	Same as diet #1
3 (Spring)	9	Same as diet #1
3 (Summer)	12	Same as diet #1
4 (All seasons)	12	Same as diet #1

Table 3. Mean (± SD) final length, mean final weight, mean feed intake, and mean weight gain on a per-fish basis for fish fed four dietary treatments during the winter and early spring period (December 13, 1999 to May 1, 2000).

Dietary treatment	Mean final length (mm)	Mean final weight (g)	Mean feed intake (g)	Mean weight gain (g)
#1. Control	144.1 ± 1.1 [a]	35.3 ± 1.5 [a]	38.5 ± 1.3	22.3 ± 1.0 [a]
#2.	142.0 ± 0.7 [b]	31.6 ± 0.8 [b]	18.2 ± 0.3	18.6 ± 1.0 [b]
#3.	136.9 ± 0.9 [c]	30.5 ± 0.4 [b]	17.7 ± 0.2	17.5 ± 0.1 [b]
#4.	146.4 ± 1.5 [a]	37.0 ± 1.1 [a]	36.5 ± 1.3	24.0 ± 0.7 [a]

Footnotes:
Initial weight and length were 13 g and 107.8 mm, respectively.
Mean values followed by the same letter are not significantly different, P<0.05.

Table 4. Mean (± SD) specific growth rate, feed efficiency ratio and protein retention on a per-fish basis for fish fed four dietary treatments during the winter and early spring period (December 13, 1999 to May 1, 2000).

Dietary treatment	Specific growth rate	Feed efficiency ratio (g gain/g feed)	Protein retention (%)
#1. Control	0.706 ± 0.016 [a]	0.558 ± 0.011 [b]	19.6 ± 2.1 [b]
#2.	0.619 ± 0.034 [b]	0.966 ± 0.067 [a]	34.1 ± 0.6 [a]
#3.	0.601 ± 0.011 [b]	0.940 ± 0.011 [a]	29.9 ± 0.4 [a]
#4.	0.746 ± 0.010 [a]	0.633 ± 0.003 [b]	15.6 ± 0.5 [c]

Footnote:
Mean values followed by the same letter are not significantly different, P<0.05.

Table 5. Mean (± SD) proximate composition (wet-weight basis) of juvenile chinook salmon fed four experimental diets at the end of the winter - early spring period (May 1, 2000).

Dietary treatment	Moisture (%)	Crude protein (%)	Crude lipid (%)	Ash (%)
#1. Control	70.7 ± 1.4 [b]	16.2 ± 1.2	10.0 ± 0.9 [a]	2.9 ± 0.7
#2.	72.5 ± 1.3 [ab]	16.2 ± 0.5	7.8 ± 1.0 [b]	2.7 ± 0.9
#3.	71.6 ± 0.4 [ab]	17.2 ± 0.1	7.9 ± 0.2 [b]	2.9 ± 0.4
#4.	73.7 ± 0.6 [a]	17.2 ± 0.4	6.0 ± 0.3 [c]	2.7 ± 0.3

Footnote:
Mean values followed by the same letter are not significantly different, P<0.05.

Table 6. Mean (± SD) nutrient content per fish (wet-weight basis) of juvenile chinook salmon fed four experimental diets at the end of the winter - early spring period (May 1, 2000).

Dietary treatment	Mean total weight (g)	Crude protein (g)	Crude lipid (g)	Ash (g)
#1. Control	35.3 ± 1.5[a]	4.8 ± 1.1	3.2 ± 0.8 [a]	0.8 ± 0.2
#2.	31.6 ± 0.8 [b]	4.4 ± 0.9	2.2 ± 0.5 [b]	0.9 ± 0.2
#3.	30.5 ± 0.4 [b]	4.5 ± 0.9	2.1 ± 0.4 [b]	0.6 ± 0.2
#4.	37.0 ± 1.1 [a]	5.8 ± 1.1	1.9 ± 0.4 [b]	0.9 ± 0.2

Footnote: Mean values followed by the same letter are not significantly different, P<0.05.

Table 7. Mean (± SD) initial weight, mean final weight, mean feed intake, and mean weight gain on a per-fish basis for fish fed four dietary treatments during the late spring period (May 1 to June 26, 2000).

Dietary treatment	Mean initial weight (g)	Mean final length (mm)	Mean final weight (g)	Mean feed intake (g)	Mean weight gain (g)
#1. control	35.3 ± 1.5[a]	164.7 ± 1.5	56.8 ± 1.9 [a]	24.6 ± 0.6	21.7 ± 1.1
#2.	31.6 ± 0.8 [b]	162.3 ± 0.7	52.5 ± 1.4 [ab]	22.0 ± 0.6	21.5 ± 0.6
#3.	30.5 ± 0.4 [b]	157.0 ± 0.6	49.5 ± 0.2 [b]	20.9 ± 0.3	19.6 ± 0.2
#4.	37.0 ± 1.1 [a]	167.3 ± 0.9	56.6 ± 2.1 [b]	24.8 ± 1.4	20.3 ± 2.1

Footnote: Mean values followed by the same letter are not significantly different, P<0.05.

Table 8. Mean (± SD) specific growth rate, feed efficiency ratio, protein retention, and final condition factor of fish fed four dietary treatments during the late spring period (May 1 to June 26, 2000).

Dietary treatment	Specific growth rate	Feed efficiency ratio (g gain/g feed)	Protein retention (%)	Fulton's condition factor	Dorsal fin height (mm))
#1. control	0.860 ± 0.018 [ab]	0.883 ± 0.021 [bc]	27.5 ± 2.1 [ab]	1.23 ± 0.02	17.0 ± 0.0
#2.	0.943 ± 0.021 [a]	0.978 ± 0.023 [a]	31.7 ± 1.8 [a]	1.24 ± 0.05	16.7 ± 0.6
#3.	0.900 ± 0.015 [ab]	0.940 ± 0.020 [ab]	25.3 ± 0.9 [b]	1.19 ± 0.03	16.3 ± 0.6
#4.	0.796 ± 0.075 [b]	0.821 ± 0.059 [c]	28.5 ± 3.0 [ab]	1.19 ± 0.02	17.3 ± 0.6

Footnote: Mean values followed by the same letter are not significantly different, P<0.05.

Table 9. Mean (± SD) proximate composition (wet-weight basis) of juvenile chinook salmon fed four experimental diets at the end of the late spring period (June 26, 2000).

Dietary treatment	Moisture (%)	Crude protein (%)	Crude lipid (%)	Ash (%)
#1. Control	72.0 ± 1.4	16.3 ± 0.9 [b]	8.2 ± 1.7[a]	3.6 ± 1.3
#2.	72.3 ± 0.9	16.7 ± 0.2 [ab]	7.8 ± 1.0[ab]	3.3 ± 0.7
#3.	73.7 ± 1.2	16.5 ± 0.1 [b]	6.3 ± 0.9 [ab]	3.3 ± 0.7
#4.	74.1 ± 0.7	17.8 ± 0.4 [a]	4.9 ± 1.1 [b]	2.0 ± 0.6

Footnote: Mean values followed by the same letter are not significantly different, P<0.05.

Table 10. Mean (± SD) nutrient content per fish (wet-weight basis) of juvenile chinook salmon fed four experimental diets at the end of the late spring period (June 26, 2000).

Dietary treatment	Mean total weight (g)	Crude protein (g)	Crude lipid (g)	Ash (g)
#1. Control	56.8 ± 1.9	9.3 ± 0.6 [b]	4.7 ± 1.1 [a]	2.1 ± 0.7
#2.	52.5 ± 1.4	9.0 ± 0.3 [b]	4.2 ± 0.6 [ab]	1.8 ± 0.3
#3.	49.5 ± 0.2	8.3 ± 0.2 [c]	3.2 ± 0.5 [ab]	1.6 ± 0.3
#4.	56.6 ± 2.1	10.3 ± 0.3 [a]	2.8 ± 0.7 [b]	1.1 ± 0.3

Footnote: Mean values followed by the same letter are not significantly different, P<0.05.

TASK 2: ASSESSMENT OF QUALITY OF FISH RELEASED FROM ONGOING CAPTIVE BROODSTOCKS

(PROGRESS REPORT: 1 JUNE 2000 THROUGH 31 MAY 2001)

by

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Introduction

Captive broodstocks are presently being reared for ESA-listed chinook salmon in Oregon and Idaho, and sockeye salmon in Idaho. A key to the success of these programs will be consistent production of juveniles with the fitness required for increased post-release survival and adult return. However, two programs are releasing juveniles into the habitat, the Redfish Lake sockeye program and the Oregon Department of Fish and Wildlife chinook program. The Redfish Lake sockeye release program has met with somewhat variable success, while the Oregon program has only recently started. A major strategy for the Redfish Lake program is release of presmolts into Stanley Basin Lakes in early fall, with the fish overwintering and outmigrating as smolts the following spring. Over the last few years (1995-1998), over-winter survival of sockeye salmon presmolts released into Stanley Basin Lakes has ranged from only a few percent to about 40%, and averaged about 20% (P. Kline, IDFG, personal communication, March 1999).

The dynamics driving over-winter survival in Stanley Basin Lakes are poorly understood. Over-winter survivals of fish released into top salmon producing systems in Canada and Alaska often average above 60%. However, Stanley Basin lakes are highly oligotrophic and are located at the highest elevation (6500 ft) and farthest distance (950 miles) from the sea of any sockeye-producing lake in North America. These factors may limit carrying capacity and absolute production thresholds. These factors may also influence the quality of fish rearing in the habitats.

Previous studies on Stanley Basin kokanee suggest that they have greatly depleted springtime lipid levels and may lack energetic reserves. It is not known if the dietary factors that affect over-wintering kokanee also affect hatchery released sockeye that must over-winter in the same habitat. Energetic reserves for fish released from the captive

broodstocks will depend on diet composition and feeding level during hatchery culture, and feeding success after release. However, drastically low energy reserves may exacerbate in-lake mortality. Low springtime lipid levels may also leave the fish lacking the energetic reserves necessary to complete downstream migration.

The consequences of inappropriate energetic reserves can be profound. There is a critical need to determine the quality of fish used for ESA-related recovery programs, both at time of release into the rearing habitat and at outmigration. It is anticipated that these measurements will provide guidance for developing rearing and release strategies to enhance survival both in the habitat and during downstream migration.

In cooperation with the Stanley Basin Sockeye Technical Oversight Committee and the Chinook Salmon Captive Propagation Technical Oversight Committee, fish were sampled in 1999 both during hatchery rearing, and at times of release into and outmigration from rearing habitats. These evaluations concentrated on sockeye salmon, but expanded to include chinook salmon in 2000 and 2001 (first sampling for 2000 occurred on 25 and 26 May 2000). Additional fish were collected in 1999 in cooperation with IDFG from various streams and these fish are being assessed for whole body energy reserves (lipid stores), fin quality, and morphometrics. Samples were taken again in 2000 by IDFG and subjected to the same analysis, and repeated once more in 2001.

Assessing relevant parameters will establish a database by which to compare rearing habitat, downstream, and adult return survival. The goal will be to determine optimum physical and biochemical benchmarks for hatchery-raised fish to increase fish survival. These measurements will aid in development of rearing strategies to enhance survival. This data will also be used to prioritize research projects in feeds and nutrition, and other culture-related areas.

Work Completed

In 1999, 103 sockeye salmon from Idaho were collected from Redfish Lake, Alturus Lake, and Sawtooth Hatchery for proximate analysis. Fish from Sawtooth Hatchery were sampled to provide a basis for comparison of hatchery pre-smolts with outmigrants from the natural systems. Fish were collected by sampling at Redfish and Alturus Lake weirs. Samples were stored at –80 ^{o}C at the UI Hagerman Fish Culture Experiment Station prior to analysis. Proximate analysis was conducted on whole bodies of the fish. Where possible, fish were analyzed individually, but in some cases, fish were too small for individual analysis and were pooled for analysis. Proximate analysis followed AOAC procedures, with the exception of lipid analysis, which was done using a LECO TFE 2000 supercritical CO_2 fat extractor. Crude protein, lipid, and ash were measured on dry samples, and data converted to a wet-weight basis using whole body moisture levels measured in each sample.

In 2000, 126 sockeye salmon were collected for analysis. In addition to collection at Redfish and Alturus Lakes, sampling was extended to Pettit Lake. Smolts and pre-

smolts (fall releases into Redfish Lake) were sampled from Sawtooth Hatchery and Eagle Hatchery, and from fish raised by NMFS at the Manchester Research Station.

Tables 1 and 2 summarize data of fish collected in 1999 and 2000, respectively. Complete proximate analysis was conducted, e.g., moisture, protein, lipid, and ash contents, and values were converted to a wet-weight basis, as noted above. However, the data shown are limited to whole body lipid content, expressed on a dry-weight basis, to highlight differences between wild and hatchery fish.

Data collected in 1999 and 2000 clearly illustrate differences in whole body lipid content between wild outmigrants and hatchery-reared outmigrants, with wild fish having twice as much lipid as hatchery fish at the time of migration. In contrast, hatchery smolts and presmolts contained more than twice as much whole body lipid as did wild fish, between 30 and 38% on a dry-weight basis. Presmolts, which are released into Redfish Lake in fall to overwinter and migrate in the spring, experienced a 5 - 6 fold decrease in whole body lipid reserves during the overwintering period. These data were confirmed in samples collected in 2000, and suggest that the practice of planting hatchery fish in the fall into Redfish Lake leads to dramatic reductions in the energy reserves of the fish prior to downstream migration, - an undesirable outcome. No major differences in whole body protein or ash content, expressed on a wet weight basis and adjusted for fish size, were found (data not shown).

Work to be Completed

Additional samples of salmon from Idaho are being collected in spring, summer, and fall of 2001. Fish quality analysis by the UI (proximate analysis, fin quality, morphometrics) will follow prescribed methods and be conducted during winter, 2002. Lipids from these fish will be analyzed for whole body fatty acid composition (conducted at the UI Hagerman Fish Culture Experiment Station). Additional samples will be analyzed for mineral content, as planned. During spring, 2002 data from all years (1999 - 2002) will be compiled, statistically analyzed, and a final project report will be written.

Table 2. Sampling dates and locations, numbers of fish sampled, and mean whole body lipid content, expressed on a percent dry weight basis, for 1999 (Task 3).

Sample Date	Sample Location	Description of Fish Sampled	Number Sampled	Mean Lipid (% Dry Wt.)
5/4/1999	ALT Trap	Wild/Natural outmigrants	1	19.4
5/4/1999	ALT Trap	Hatchery-produced outmigrants	2	10.6
5/27/1999	ALT Trap	Direct release	9	5.8
5/27/1999	ALT Trap	Wild/Natural outmigrants	5	12.2
5/4/1999	RFL Weir	Wild/Natural outmigrants	4	10.6
5/4/1999	RFL Weir	Smolt hatchery outmigrant	5	5.9
5/26/1999	RFL Weir	Wild/Natural outmigrants	5	9.2
5/26/1999	RFL Weir	Unknown	3	15.5
5/26/1999	RFL Weir	Direct release	12	5.6
5/27/1999	RFL Weir	Net pen release	12	9.8
5/4/1999	Sawtooth Hatchery	Smolts (NMFS)	10	36.8
5/4/1999	Sawtooth Hatchery	Smolts (Eagle)	10	37.8
10/7/1999	Sawtooth Hatchery	Hatchery pre-smolts	25	30.8

Table 1. Composition of experimental diets for chinook salmon captive broodstock studies in Task 2[1].

INGREDIENT	DIETS 1 & 2 (%)[1]	DIET 3 (%)[1]
Anchovy meal	54.00	54.00
Blood meal	4.00	4.00
Wheat gluten	4.00	4.00
Wheat mids	16.00	12.50 to 19.50
Fish oil	13.00	6.00 to 13.00
Vitamin C	0.30	0.30
Choline	0.50	0.50
TM salt[2]	0.10	0.10
Vitamin premix[3]	3.00	3.00
Krill hydrolysate	5.00	5.00
Astaxanthin[4]	0.10	0.10

Footnotes:
1. Calculated Proximate Composition of Diets 1 & 2 = 48% crude protein (45% digestible protein) and 17.5% crude lipid, expressed on an "as-is" basis (7% moisture). Diet 3 varies to keep energy intake constant while varying daily protein intake (g protein/kg fish/d).
2. Supplies the following per kg dry diet: Zn as ZnSO4.7 H2O, 75 mg; Mn as MnSO4.H2O, 20 mg; Cu as CuSO4.5 H2O, 1.54 mg; I as KIO.C2H8N2 .2HI, 10 mg.
3. Supplies the following per kg dry diet: thiamin mononitrate, 62 mg; riboflavin, 71 mg; niacin, 294 mg; calcium pantothenate, 153 mg; pyridoxine hydrochloride, 50 mg; folic acid, 22 mg; vitamin B12, 0.08 mg; d-biotin, 0.8 mg; myoinositol, 176 mg; retinol acetate, 8818 IU; vitamin D3, 588 mg; (-tocopherol acetate, 670 mg; menadione sodium bisulfite complex, 37 mg.
4. Carophyll Pink, Hoffman La-Roche, Basle, Switzerland.

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Sample Date	Sample Location	Description of Fish Sampled	Number Sampled	Mean Lipid (% Dry Wt.)
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5/27/1999	ALT Trap	Direct release	9	5.8
5/27/1999	ALT Trap	Wild/Natural outmigrants	5	12.2
5/4/1999	RFL Weir	Wild/Natural outmigrants	4	10.6
5/4/1999	RFL Weir	Smolt hatchery outmigrant	5	5.9
5/26/1999	RFL Weir	Wild/Natural outmigrants	5	9.2
5/26/1999	RFL Weir	Unknown	3	15.5
5/26/1999	RFL Weir	Direct release	12	5.6
5/27/1999	RFL Weir	Net pen release	12	9.8
5/4/1999	Sawtooth Hatchery	Smolts (NMFS)	10	36.8
5/4/1999	Sawtooth Hatchery	Smolts (Eagle)	10	37.8
10/7/1999	Sawtooth Hatchery	Hatchery pre-smolts	25	30.8

Table 3. Sampling dates and locations, numbers of fish sampled, mean weight and fork length, and mean whole body lipid content, expressed on a percent dry weight basis, for 2000 (Task 3).

Sample Date	Sample Location	Description of Fish Sampled	Number Sampled	Mean Wt. (g)	Mean F. L. (mm)	Mean % lipid Dry Wt.
5/22/2000	RFL Weir	Hatchery-produced outmigrants	20	10.8	112	3.6
5/22/2000	RFL Weir	Wild/Natural outmigrants	8	16.7	128	11.8
5/23/2000	ALT Screw Trap	Hatchery-produced outmigrants	3	10.2	113	0.6
5/23/2000	ALT Screw Trap	Wild/Natural outmigrants	4	4.4	86	5.7
7/27/2000	Eagle Hatchery	Hatchery pre-smolts	20	7.8	95	9.3
7/27/2000	Sawtooth Hatchery	Hatchery pre-smolts	20	2.2	60	28.7
9/25/2000	Alturas Lake	Trawl Samples	18	–	–	22.8
9/29/2000	Redfish Lake	Trawl Samples	8	–	–	16.8
9/28/2000	Pettit Lake	Trawl Samples	4	18.8	126	34.0
9/28/2000	Pettit Lake	Trawl Samples	1	51.5	180	31.0
10/11/2000	Sawtooth Hatchery	Hatchery pre-smolts	20	11.1	105	33.4

TASK 3. DETERMINE CRITICAL IMPRINTING PERIODS FOR SOCKEYE SALMON

(PROGRESS REPORT: 1 JUNE 2000 THROUGH 31 MAY 2001)

by

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Introduction

Pacific salmon are well known for their ability to learn (or imprint) to odors associated with their natal stream as juveniles and then later use these retained odor memories to guide the final phases of their home-stream migration. The imprinting process is critical for successful completion of the spawning migration and salmon that do not experience their natal water during appropriate juvenile stages are more likely to stray to non-natal sites. Straying by captively-reared salmon may jeopardize efforts to enhance endangered populations by either lowering the effective number of spawning adults in a captively-reared target population or via competition and interbreeding of hatchery-reared salmon with endangered wild populations.

Experimental evidence has indicated that olfactory imprinting in coho salmon occurs during a sensitive period associated with a surge in plasma thyroxine levels during the parr-smolt transformation (Dittman et al. 1996). However, the freshwater rearing patterns of sockeye salmon are much more complex and the critical periods for imprinting are not known. In sockeye salmon, the parr-smolt transformation may occur downstream or in a different location from incubation and early rearing habitats (Groot and Margolis 1991), yet salmon migrate past areas where they had undergone the parr-smolt transformation and return to within a very close proximity of their natal habitat. Captive rearing programs often occur at locations which do not provide the same water source the fish would experience in its natural habitat, and therefore the timing of releases may influence homing ability. Juvenile release strategies for captively-reared sockeye salmon must not only maximize survival but also minimize subsequent adult straying by ensuring that juveniles have successfully imprinted. The timing of imprinting

and the effects of artificial incubation and early rearing environments on imprinting must be determined before release strategies that minimize straying can be developed.

The experiments to determine the critical period(s) for olfactory imprinting by sockeye salmon were initiated in fall 2000. After emergence from their natal gravel, sockeye salmon migrate to or remain in a lake, where they rear for 1 or 2 years before smolting and migrating to sea. To determine the relative importance of odorant exposure during these key developmental periods, sockeye salmon are being exposed to specific odorants as alevins/emergent fry (the period just prior to and during emergence from the natal gravel - February, 2001) or as smolts (March - May, 2002). Assessment of imprinting will be conducted in fall 2002 and 2003 by measuring olfactory sensitivity to exposure odorants using behavioral assays and electro-olfactograms (EOG), a relatively simple electrophysiological technique used extensively in fishes to measure the sensitivity of the olfactory epithelium to specific odorants (Hara 1992, Sorensen and Caprio 1997). EOG responses reflect the summated responses of many receptor neurons in the olfactory epithelium (Ottoson 1971). Because the olfactory epithelium is apparently sensitized to specific odors during imprinting (Nevitt et al. 1994, Dittman et al. 1997), the EOG may provide a rapid, sensitive, and cost-effective method for assessing sensitization to imprinted odorants.

Work Completed

Four thousand Columbia River sockeye salmon were obtained from the Colville Tribe Cassimer Bar Salmon Hatchery (eyed eggs) in November 2000. Embryos were transferred to the Northwest Fisheries Science Center and reared in chilled dechlorinated Seattle City water. Fish were divided into three treatment groups: 1) alevins/emergent fry exposure, 2) smolt exposure, and 3) control. The smolt exposure group will be further divided into three groups with different exposure lengths.

The alevins/emergent fry were continually exposed to a mixture of imprinting odorants (phenylethyl alcohol (PEA), L - Arginine, L - Threonine, and L – Glutamate) at a final concentration of 100 nM each from February 1 to March 5, 2001. PEA has been used extensively as an odorant for studying imprinting (Hasler and Scholz 1983, Nevitt et al. 1994, Dittman et al. 1996, 1997). Amino acid odorants have also been used in imprinting studies (Morin et al. 1989) and the three amino acids used in this study represent potent odorants that activate distinct receptor types in the olfactory epithelium (Hara 1992). The use of these odorants anticipates the future development of new molecular assays for olfactory imprinting (see below).

A functional EOG apparatus/lab has been constructed and is being tested for imprinting assays.

Work to be Completed

Fish will be moved to the University of Washington's Big Beef Creek Research Station in June - July 2001 and maintained there until the end of the experiment. Beginning in February 2002 until June 1, 2003, 12 fish/treatment from each exposure group will be sacrificed every three weeks for physiological sampling of gill Na^+/K^+ ATPase activity (McCormick 1993) and plasma thyroxine (Dickhoff et al. 1982) to assess smolting. To assess how long fish need to experience their natal water prior to release, the smolt exposure groups will be continuously exposed to the imprinting odorants for 12 weeks (March 1 – May 24, 2002), 6 weeks (April 12 – May 24, 2002), or 3 weeks (May 3 - May 24, 2002). All groups will be maintained separately until after the parr-smolt transformation (May 31, 2002), then marked by treatment and reared communally to maturity.

Development and testing to ready the EOG apparatus for testing odor-exposed sockeye will occur throughout the fiscal year 2001-2002. During fall 2002, 10 fish/treatment group will be tested for heightened EOG sensitivity to the exposure odors relative to reference odorants. Differences between treatment groups in EOG responsiveness to specific odorants will be examined by analysis of variance (ANOVA) followed by Fisher's PLSD. Significance for all analyses will be established at the $P<0.05$ level. Olfactory rosettes from all sacrificed fish will be collected and frozen for later analysis of odorant receptor mRNA levels. This molecular approach to assay for olfactory imprinting is being developed as part of another project but may be easily adapted to this study if successful.

There is evidence that salmon must undergo sexual maturity to demonstrate heightened olfactory sensitivity and behavioral attraction to imprinted odors (Hasler and Scholz 1983, Dittman et al. 1997). Therefore the majority of the behavioral and EOG evaluations of olfactory imprinting will be conducted in fall 2003 when these fish are expected to mature. While a few early maturing males (2-20% in captively-reared Redfish Lake sockeye salmon; W. McAuley, NMFS, personal communication) will be available in September 2002 to test for the importance of maturation for recognition of imprinted odorants, the requirement for significant numbers of maturing fish (and therefore full-life cycle rearing) to test for imprinting is a major obstacle for development of routine assays for imprinting. Recent studies suggest that the maturational hormone GnRH can stimulate migratory behavior in homing salmon (Dittman, unpublished) and heighten olfactory sensitivity (Eisthen et al. 2000). Using coho salmon exposed to imprinted odorants during smolting (March - May 2001), the known critical period for imprinting in hatchery-reared coho, the efficacy of GnRH analog implants for inducing EOG olfactory sensitivity to imprinting odorants in odorant-exposed fish will be tested. If successful, GnRH implants will also be used for EOG evaluations of odorant-exposed sockeye in fall 2002 to determine the timing of imprinting in the experimental sockeye salmon.

Data analysis and final reports for this Task will be completed in 2004.

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TASK 4. EVALUATE THE EFFECTS OF EXERCISE TRAINING AND EXOGENOUS HORMONES ON REPRODUCTIVE PERFORMANCE, AND ASSOCIATED CHARACTERISTICS

(FINAL REPORT: 1 JUNE 2000 THROUGH 31 MAY 2001)

by

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Introduction

Pacific salmon (*Oncorhynchus* spp.) reared to adulthood in captivity for recovery of imperiled populations experience dramatically different environments than those in which their ancestors evolved. In addition to being fed formulated feeds, captively-reared salmon expend less energy swimming than wild salmon during their extensive spawning migrations. Upon returning to their river of origin, wild salmon cease feeding from days to several months prior to the onset of reproductive activity. During upstream migration and spawning fat reserves are utilized first, followed by the utilization of muscle (i.e., protein). Energy reserves are used for migration, the development of secondary sex characteristics (Hendry and Berg 1999), and for intrasexual competition, nest construction, nest guarding and other reproductive behaviors. The relationships among energy expended for swimming, reproductive activity, and concurrent endocrine changes during final maturation (Slater et al. 1994) are poorly understood, but may be important in explaining the generally poor reproductive performance exhibited by captively-reared salmon (e.g., Berejikian et al. 2001a,b).

Captive rearing to adulthood can substantially affect reproductive behavior and estimated reproductive success of salmon spawning naturally (Berejikian et al. 1997, Fleming et al. 1996). Although direct comparative studies have not been conducted with wild chinook salmon, captively-reared chinook salmon females frequently abandon their nests without spawning, deposit a low percentage of their eggs into the gravel, and males often exhibit little courtship response to nearby nest-digging females (Berejikian et al. 2001b). In addition, eggs of adult captively-reared chinook salmon spawning in natural streams suffer high mortality. Fertilization rates are lower and post-fertilization mortality is higher than exhibited by naturally spawning wild fish or captively-reared fish that are spawned artificially (P. Kline, Idaho Department of Fish and Game, personal communication). Identifying relationships between environmental conditions salmon experience in captivity and endocrine changes required for successful natural spawning may help guide improvements in husbandry practices. The approach of the present study was to manipulate one environmental factor (swimming activity), and one endocrine factor (GnRHa levels) to evaluate their independent and combine effects on reproductive performance.

The large body of published literature on the effects of current velocities in fish culture systems suggests that exposing fish to moderate 'exercise' improves physical fitness. For example, exercise improves growth rates in brown trout, *Salmo trutta* (Davidson and Goldspink 1977), brook char, *Salvelinus fontinalis*, (East and Magnan 1987), rainbow trout, *O. mykiss* (Houlihan and Laurent 1987), and Atlantic salmon, *S. salar* (Totland et al. 1987). Improved growth in exercised fish may be caused by behavioral effects, such as reduced aggression (Adams et al. 1995) or increased feed conversion (Leon 1986). Fitness-related parameters of salmon health, including fin quality (Joergensen and Jobling 1993), muscle hypertrophy (Barret and McKeown 1988), and swimming stamina (Leon 1986, McDonald et al. 1998), have also been improved through moderate exercise. However, exercise has apparently not produced beneficial

effects for juvenile chinook salmon (Kiessling et al.1994, White and Li 1985), and studies measuring the potential effects of sustained moderate exercise on adult reproductive characteristics of chinook or other salmon have not been published. Therefore the first hypothesis proposed is that reported general improvements in physical fitness found in numerous salmonids would occur in maturing chinook salmon and would benefit them during the physically demanding reproductive phase of their life-cycle.

Wild salmon undergo dramatic hormonal changes during the final maturation (e.g., Slater et al. 1994). Environmental stimuli, including temperature (Davies and Bromage 1991) and photoperiod (Taranger et al. 1998), influence the process of final maturation. Other environmental characteristics in artificial culture systems (current velocity, density, etc.), which differ from those experienced by salmon in natural environments, may inhibit natural hormonal processes necessary for optimal reproductive success.

To determine whether hormonal factors may be driving the reproductive limitations of captively-reared chinook salmon, the effects of exogenous GnRHa hormone on natural reproductive performance were investigated. GnRHa was selected because it is the central regulator of reproduction in vertebrates, including fish. GnRH in the brain stimulates the release of gonadotropin in the pituitary (Kobayashi et al. 1997), which in turn stimulates gonadal sex steroid production, gametogenesis, and gamete release. Numerous studies have shown that injection or implants containing GnRH analogues induce ovulation and spermiation in salmonids (Mylonas et al. 1992). In addition, GnRHa implants in Pacific salmon has been demonstrated to shorten homing duration (Kitahashi et al. 1998, Sato et al. 1997) and increase homing precision (A. Dittman, NMFS, unpublished data). Therefore the second hypothesis is that other behaviors associated with successful natural spawning (e.g., appropriate timing, nest construction, aggression) would also be improved if, in fact, the aforementioned behavioral deficiencies ofcaptively-reared salmon are hormone-related.

Methods and Materials

The study population

Chinook salmon eyed embryos were hydraulically sampled from the nests of wild fish which spawned in the Dungeness River, WA in December 1996. The embryos were transported to the Washington Department of Fish and Wildlife (WDFW) Hurd Creek Hatchery (Sequim, WA) where they were incubated and the fry subsequently reared in fresh well-water. The emergent fry were initially stocked into 1.2-m diameter circular tanks in February 1996, held for 6 months, then transferred (at mean weight, 21 g) to 6-m diameter tanks (35.4 m^3), where rearing density did not exceed 2.3 $kg \cdot (m^3)^{-1}$. During the freshwater- rearing phase, the fish were fed a commercial semi-moist diet (BioOregon[1], Warrenton, Oregon). In April 1998, 240 fish were transferred to the National Marine Fisheries Service (NMFS) Manchester Research Station for rearing in seawater.

[1] Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

Rearing treatments

On 27 October 1999, 128 chinook salmon were divided equally into 4 indoor tanks (diameter = 4.1 m, depth = 1 m) to create 2 replicate tanks each for the high velocity (HV) and low velocity (LV) treatments. Each tank received *ca*. 110 $L \cdot min^{-1}$ seawater (temperature 8.3 – 13.6 °C; *ca.* 28 ppt salinity), which was treated by filtration (<5 μm) to remove suspended solids and disinfected with ultraviolet light. Flow into each tank was directed through a nozzle placed parallel to the surface to create a circular current. Water current velocity was increased in the two HV tanks by centrifugal pumps equipped with timer-controlled variable frequency drives. The recirculated water re-entered the tank under high pressure through a submerged nozzle located at the tank perimeter. Water was not recirculated in two LV tanks. Water velocity was periodically measured with a Swoffer[1] flowmeter (Swoffer Instruments, Inc., Seattle, WA) along concentric circles 0.3 m, 1.2 m, and 1.8 m from the tank center, at planes 30 cm above the tank bottoms, and simple arithmetic means of the measurements calculated. In the LV and HV tanks, maximum velocity averaged 18.4 $cm \cdot sec^{-1}$ and 55.8 $cm \cdot sec^{-1}$, respectively during the majority of experimental rearing period (Figure 1). The resulting velocity patterns, i.e., highest velocities near the wall and lowest velocities near the center standpipe, were similar to that in other studies investigating the effects of current velocity in circular tanks (McDonald et al. 1998; Skilbrei and Holm 1998). All fish experienced a natural photoperiod.

Fish in the HV tanks were acclimated to the experimental rearing conditions by gradually increasing both water current velocity and duration of exposure. Between 27 October and 12 December 1999 all groups of fish were maintained under LV conditions. Water velocity was increased in the HV tanks to about 90% of maximum for 4 $h \cdot d^{-1}$ between 13 December 1999 and 2 January 2000, and then to 16 $h \cdot d^{-1}$ until 25 January 2000. Fish in the HV tanks were continuously exposed to maximum water current velocities between 26 February and 11 July 2000 (Figure 1). Fish in each tank were weighed (0.1 g), measured (1.0 mm fork length), and counted at the start of the trial (27 October 1999), and on 18 February and 4 May 2000. The fish were fed to apparent satiation on a daily basis via timer-controlled automatic feeders with a high energy (ca. 25% crude lipid) commercial broodstock diet (Moore-Clark[1], Inc., Vancouver, B.C., Canada) until 1 May 2000, when feeding was terminated.

Maturing adults were transferred to two 5-m diameter freshwater holding tanks between 11 July and 17 July 2000. Each freshwater holding tank received equal numbers of fish from each of the four seawater-rearing tanks. The holding tanks received approximately 80 $L \cdot min^{-1}$ of recirculated water (10 - 12 ˚C) from the stream channel where the reproductive behavior experiment was conducted. Thus, mature fish from both HV and LV treatments were held in common low-velocity (approximately 10 – 20 cm/s) tanks for 6 to 8 weeks prior to spawning. Fish not used in the experiment were returned to the Dungeness Hatchery and spawned artificially.

Seventy-three of the 128 fish matured (65 females and 8 males) in 2000. On 28 August 2000 all fish were anesthetized (tricane methane sulfonate), weighed, measured

(fork length), and a numbered Peterson Disk tag (2.5 cm) was attached to each fish for individual identification. None of the females had ovulated as of 28 August (i.e., eggs could not be expressed and bellies were not soft).

Groups of females were released into the experimental channel (see below) on two occasions. For the first release (release 1), 8 females from each velocity treatment (16 total; 4 per tank) were selected to equalize mean body size among tanks. On 29 August two of the four females from each of the four tanks (eight total) were treated with ethylene vinyl acetate co-polymer (EVAC) implant(s) containing GnRHa (Reproboost®, Lot #s EV75-061598 and EV150-041699, purchased from Veripharm, Inc., Baltimore, MD[1]) by intramuscular injection. Dosages were approximately 0.075 μg ·body weight $(g)^{-1}$. The remaining eight females were not implanted. Implanted fish are hereafter referred to as IMP and non-implanted as NIMP. Females in release 1 were stocked into the stream channel with all eight males (5 LV and 3 HV) on 2 September 2000. For release 2, 16 females were selected in the same manner as release 1; eight females were implanted on 11 September and all 16 were stocked on 15 September 2000. None of the eight males was implanted with GnRHa. The 14 days between release 1 and 2 was made to reduce the number of females which would be sexually active at any one time.

Spawning channel configuration

The spawning experiment was conducted in a 40 x 6 m stream channel located at the NMFS Manchester Research Station (Berejikian et al. 2001b). Briefly, 6800 $L \cdot min^{-1}$ was recirculated through the channel, with 60 $L \cdot min^{-1}$ flow through. Water depth (10 to 35 cm), current velocities at nest sites (0.0 and 0.5 m), water temperature (9.9 - 12.2 °C), and gravel size (approximately 3 – 12 cm diameter) were within the range of spawning habitat reportedly used by several chinook salmon populations in the Dungeness River. Four flood lights positioned 4.5 m above the channel were left on continuously and were directed horizontally to produce enough light at the water surface (mean $\pm$ SD = 4.8 $\pm$ 0.9 lux) for night-time video recording (see below).

Behavioral observations

Reproductive behavior of fish in the stream channel was recorded by three methods: (i) direct 'scan' sampling, (ii) overhead video cameras connected to time-lapse recorders, and (iii) underwater video recording. The behaviors quantified were categorized into four classes: nesting behavior, aggression, reproductive status, and duration (Table 1). To conduct the scan sampling, fish in the stream channel were viewed from an observation blind between 0700 and 1900 h. Each fish in the channel was observed (scanned) for 10 minutes twice daily. The frequency of nest digs, covering digs, and probes (see Table 1 for definitions) were recorded. Two types of aggressive behaviors, attacks, and threat displays were recorded along with the initiator and receiver of each aggressive act (Table 1). Aggressive behavior definitions followed those described in Tautz and Groot (1975) and Berejikian et al. (1997). The response variable for each behavior was calculated as the sum of frequencies divided by the number of scans (i.e., frequency per 10 min observation).

Alpha males were those that dominated access to an ovipositing female, and were first to enter the nest and release milt during oviposition. Beta males were subordinate, and 'sneaked' into the nest and released milt during ovisposition.

Four cameras (Watec[1], model 902HS; 00015 lux sensitivity @ F1.4) each fitted with a 2.6 mm lens were positioned approximately 3.8 m above the stream channel, so that each camera captured the image of one quarter of the channel. The video signals were continuously recorded on time-lapse recorders (Gyyr[1], model TLC 2124-GY) at approximately 5 frames/second each night between 1700 hrs and 07300 hrs. All spawning events occurring at night were recorded and observed upon reviewing the video tapes between 0800 and 1200 hrs the following day.

During daylight, three remote underwater cameras were used to record spawning events. When a female appeared to be preparing a new nest for spawning, a camera was positioned nearby, and the video signals were recorded in real time (JVC[1] Super VHS, model HR S-7300-U). In a few cases the positioning of the camera disturbed the fish, but females usually returned to their nest in less than 15 minutes.

The combination of the three observation techniques allowed quantification of three reproductive phases: (i) pre-spawning duration, (ii) duration of sexual activity, and (iii) senescence, along with the number of nests spawned by each female, and the location of each nest (Table 1).

Egg deposition estimates

The ability of females to deposit eggs before death is a primary measure of breeding success (Healey 1991; Fleming and Gross 1993, 1994). To estimate the number of eggs deposited by each female, the number of eggs retained in the body cavity at death was subtracted from their estimated fecundity. Fecundity of fish spawning in the channel was estimated by entering their weight into a weight– fecundity regression equation (fecundity = 0.852 * weight (g) + 545.4; $R^2 = 0.72$, $n = 29$, $P < 0.001$) derived from cohorts reared in the same environments and spawned artificially at the Dungeness Hatchery.

Body composition

Body proximate composition analyses were performed on all chinook salmon that spawned in the stream channel (32 females and 8 males). These fish were removed from the channel when they were near death (i.e., they could no longer maintain an upright swimming posture and were drifting downstream). Some fish died over night and were collected the following morning. An additional 29 females were spawned artificially within a week of ovulation between 19 September 10 October 2000. All eggs were removed from naturally spawning and artificially spawned females, and each whole carcass was placed in an individual plastic bag, and frozen (-20 °C) until analyzed.

Individual fish were prepared for analysis by repeatedly grinding the whole, partially thawed carcasses in a silent cutter (Hobart Corp[1].) until a smooth puree was obtained. Proximate analysis of fish samples was conducted using standard (AOAC

1990) methods as follows: moisture (sec. 930.15) by oven drying to constant weight (16 h) at 105 °C, protein by nitrogen determination using a LECO FP 428 nitrogen analyzer, crude lipid (sec. 920.39C) by Soxhlet extraction with dichloromethane, and total ash (sec. 923.03).

Statistical analyses

Reproductive behavior: Prior to analyzing the effects of the independent variables of interest (current velocity, hormone, and release group) on female reproductive behavior, a one-way nested ANOVA (tanks nested within current velocity) was performed to determine whether individual seawater rearing tanks affected any of the dependent variables. Where nested tank effects were non-significant, three-way ANOVAs were performed for each dependent variables. The main effects were current velocity (HV and LV), hormone (IMP and NIMP), and release group (first and second). For variables in which ANOVA assumptions of normality and homogeneity of variance were not met, data were transformed by either logarithmic or square-root transformations. To meet the ANOVA assumption of independence, a correlation matrix of the dependent variables was constructed to identify auto-correlated dependent variables. The treatment of correlated dependent variables is described in the Results section. A Bonferroni procedure (Sokal and Rohlf 1996) was performed on the univariate ANOVAs to an overall error rate of $\alpha = 0.05$ for behavioral tests.

The effects of current velocity on time to male onset of spawning and number of participations in spawning were analyzed by two-sample t-tests. A 2 x 2 contingency table analysis was conducted to determine whether the number of beta male spawning participations was independent of the rearing treatment experienced by the alpha male.

Growth and body composition: Proximate composition dependent variables (percents lipid, ash, and protein) and growth data were analyzed by nested two-way ANOVA. The main effects were current velocity (HV or LV), and method of spawning (natural or artificial). Individual seawater rearing tanks were nested within the main effect of current velocity.

Results

Behavior of chinook salmon in rearing treatments

Fish in LV and HV tanks distributed themselves in the lower two-thirds of the water column, and generally remained within 1 m of the outer wall of the tanks. However, the vertical distribution of fish changed depending on light level - fish observed in early morning were closer to the surface, then moved deeper as light intensity increased. Fish in the HV tanks were more densely aggregated, with the fish frequently changing places and apparently "drafting" off one another. Fish in both treatments seemed to avoid the low velocity area in the center of the tank. Thus, current velocities at the 1.2 m contour (Figure 1) probably best reflect the velocities experienced by fish in both treatments.

Reproductive behavior and success

Correlated behaviors-- Two status variables, nest digging and courted, were each significantly correlated with two other frequency variables and were not considered in the three-way ANOVA (Table 2). The frequency of two nesting behaviors, nest digs and probes, were significantly correlated (Table 2) and were combined to create the dependent variable 'nest building' frequency (females often alternate probing and digging during nest construction). Post-release lifespan was positively correlated with pre-spawning duration (Table 2); 82% of the variability in post-release (i.e., reproductive) lifespan was attributable to pre-spawning duration (Figure 2), and therefore not attributable to spawning duration or post-spawning lifespan. Thus, the time from release into the spawning channel to death in these spawning fish was predominantly determined by their onset of spawning. Once a female had spawned in her first nest, time to death was fairly constant (95% C.I. = 8.2 d – 10.3 d, n = 32).

Effects of current velocity and GnRHa on reproductive behavior-- Rearing tanks, nested within the main effect of current velocity, had no significant effect on any of the variables measured ($P > 0.05$ for all tests). There were no significant two- or three-way interactions among the main effects of hormone, current velocity, and release group on any of the dependent variables ($P > 0.05$ for all tests).

The 32 females placed in the spawning channel participated in 161 spawning events (mean = 5.0, range = 1 to 8). The number of spawning events was not significantly affected by either the hormone ($F_{1,24} = 0.03$, $P = 0.99$), current velocity ($F_{1,24} = 2.79$, $P = 0.108$) or release group ($F_{1,24} = 1.24$, $P = 0.276$). The highest mean percent egg deposition was in the HV-IMP group (99.6%), and the lowest in the LV-NIMP group (78.7%). However egg deposition was not significantly affected by the main effects of current velocity ($F_{1,24} = 1.96$, $P = 0.175$), hormone ($F_{1,24} = 2.187$, $P = 0.152$), or release group ($F_{1,24} = 1.347$, $P = 0.257$). An total estimated 118,090 eggs were deposited, and 7,060 emergent fry were recovered, which equates to a 6.0% egg-to-fry survival.

Females treated with GnRHa (IMP) took significantly less time to construct and spawn in their first nest than NIMP females (Figure 3a). The IMP and NIMP treatments did not differ in their duration of sexual activity, or time from spawning their last nest until death (Table 3). The spawn timing of IMP females resembled that of naturally spawning wild fish in the Dungeness River more closely than did the spawn timing of NIMP females (Figure 4). Females treated with GnRHa were observed to be nest guarding significantly more often than NIMP females (Table 3, Figure 3b). Females treated with GnRHa also exhibited greater levels of aggression towards males than NIMP females (Table 3, Figure 3c). There were no significant main effects on any of the other dependent variables (Table 3).

Individual males participated in between 5 and 51 spawning events. Males reared in HV tanks averaged 21.0 participations as the alpha male compared with 18.4 for LV-reared males ($t = 0.21$, 4 df, $P = 0.85$). Males from HV tanks averaged 3.7 participations as the beta male compared with 3.4 for LV-reared males ($t = 0.11$, 4 df, $P = 0.92$). The proportion of spawning events in which beta males entered the nest and ejaculated at the

time of spawning was significantly greater when LV-reared males were dominant (28.2%) than spawning events in which HV-reared males were dominant (1.8%; X^2 = 5.24, 1 df, P = 0.023). In other words, HV-reared males more completely monopolized access to spawning females. Males reared in the HV tanks participated in their first spawning event sooner (2.9 d) than LV-reared males (5.3 d: t = 2.94, 4 df, P = 0.041).

Growth-- Between October and May fish in both current velocity treatments grew at similar rates, increasing 61% in weight ($F_{1,54}$ = 0.90, P = 0.34) and 18% in length ($F_{1,54}$ = 0.82, p = 0.37). During the final 5 weeks of rearing in seawater fish in both groups decreased in body mass by about 10% and increased in body length by about 3%. There were no significant differences in either length ($F_{1,54}$ = 0.51, p = 0.47) or weight ($F_{1,54}$ = 0.57, P = 0.45) at the end of the sea-water rearing phase.

Body composition-- Percent lipid was significantly affected by tanks within treatments (nested effect: $F_{2,54}$ = 5.309, P = 0.008). Nevertheless, females reared in HV tanks had significantly lower percent lipid than females reared in LV tanks ($F_{1,54}$ = 10.69, P = 0.002, Figure 5a). There were no significant nested tank effects on percent ash ($F_{2,54}$ = 0.312, P = 0.734) or protein ($F_{2,54}$ = 1.425, P = 0.249), and current velocity did not significantly affect ash ($F_{1,54}$ = 0.218, P = 0.642), or protein ($F_{1,54}$ = 0.981, P = 0.326) content of females (Figure 5a). There was a significant reduction in whole body lipid ($F_{1,54}$=15.905, P < 0.001) and protein ($F_{1,54}$ = 97.849, P < 0.001), but not ash ($F_{1,54}$ = 3.634, P = 0.062), in females that spawned naturally in the channel compared with those artificially spawned at the Dungeness hatchery (Figure 5c). There were no significant spawning location by current velocity interactions (P > 0.05 for all tests).

Males reared in HV tanks had nearly 10-fold reduction in whole body lipid content, compared with LV-reared males, but the difference was not statistically significant (t = -1.74, 6 df, P = 0.130; Fig 5 b). Males reared in HV tanks had a significantly lower percent protein content than LV-reared males (t = -2.496, 6 df, P = 0.046), and no difference was detected for ash content (t = 0.34, 6 df, P = 0.75; Fig. 5b).

Discussion

Treatment with GnRHa affected several reproductive behaviors of females including: greater degree of nest guarding, earlier onset of spawning, and greater aggression towards males. Current velocity had no apparent effect on any of the female reproductive behaviors measured. Males reared under high current velocities spawned earlier, and alpha males reared in HV tanks better defended their access to spawning females than alpha males reared in LV tanks. However, the results for males should be considered preliminary because of the small number of males available in the study. Rearing in HV tanks caused females to have lower percent lipid content and males to have lower percent protein content at the time of spawning.

These results are the first to demonstrate effects of GnRHa implants on reproductive behavior of salmon. In salmon, GnRH acts on the pituitary gland to stimulate production of gonadotropin hormone I (GTH I) during vitellogenesis and

spermatogenesis and GTH II at spawning (Swanson 1995). The effects of GnRH could be via the brain or 'downstream' bias increases in sex steroids.

Injection of GnRHa implants had clear effects on the reproductive behaviors of females. Less certain is whether the changes affected by the GnRHa implants produced reproductive behavior patterns more closely resembling those of wild fish. However, at least two of the three behaviors affected would likely improve reproductive performance under natural conditions. First, nest guarding is a mechanism by which females can protect their eggs from being dug up or physically shocked (Jensen and Alderdice 1989) and may significantly reduce mortality (Hayes 1987, see also Fleming and Gross 1994). GnRHa-implanted females were observed to be guarding their nests more than twice as often as non-implanted females. Chinook salmon, because they are semelparous, do not suffer costs of nest defense (e.g., injury) to future reproduction. In addition, the present study indicated that the greater nest guarding activity was not associated with either a decrease in nest guarding duration or an advancement in time of death. Thus, more active nest guarding should improve reproductive success through increased survival of the progeny, without negative trade-offs.

Second, the earlier onset of spawning exhibited by GnRHa-treated females is consistent with previous studies documenting advancement and synchronization of spawn timing in artificially spawned salmon from captive broodstocks (e.g., Breton et al. 1990, Slater et al. 1995). The onset of spawning of IMP females created a temporal profile similar to that of wild fish spawning in the Dungeness River and should provide an advantage to the offspring under natural conditions. Salmon populations have locally adapted spawn timing, which increases the chances that offspring will emerge from the gravel under favorable environmental conditions (e.g., temperature, food availability). For example, Einum and Fleming (2000) demonstrated selective mortality on Atlantic salmon fry produced from eggs in which fertilization was artificially delayed by 10 – 12 days. The range of accumulated temperature units (days x °C) required for embryonic development is narrow within populations of chinook salmon (Billard and Jensen 1996). Thus, delayed spawning of captively-reared chinook salmon (released as adults) during a declining temperature regime further delays emergence timing, may affect the condition of emerging fry (Beer and Anderson 2001), and magnifies the disparity between emergence timing of their offspring and that to which the population is locally adapted.

The effects of higher levels of aggression towards males are less clear than those on breeding success. Aggression towards males may represent a form of female mate selection (Maekawa et al. 1994; Berejikian et al. 1997), which in this study might suggest that IMP females were more discriminating in their choice of mates than NIMP females. Alternatively, the elevated hormone levels may have created unnaturally high levels of aggression which could interrupt nest construction and courtship, although there were no apparent delays in spawning duration of IMP females concomitant with their higher levels of aggression towards males.

Even though the behavioral changes affected by the GnRHa implants would likely improve reproductive success, it is not recommended that exogenous hormones are used

as a tool in artificial propagation programs in which captively-reared adults are being released for natural spawning (e.g., IDFG 1996). A longer-term solution to problems such as delayed spawning may lie in determining the environmental factors of captive rearing which cause reproductive abnormalities, and whether modifications to rearing practices can improve natural reproductive performance.

Although no other published studies have investigated the effects of sustained high current velocities (exercise) on maturing salmon, the negligible effects of exercise on reproductive performance and proximate composition are consistent with other studies of non-maturing chinook salmon which fail to show beneficial effects of exercise (White and Li 1985, Keissling et al. 1994). There are numerous possible reasons why no effect, positive or negative, was demonstrated in this study. First, the current velocities may have been inadequate to improve the parameters measured. The fish in the HV treatment were experiencing average current velocities of approximately 0.5 to 0.8 blps depending on their size and location in the tank. Several studies demonstrating physiological effects of sustained exercise at moderate to high (> 1 blps) have failed to show effects at low (< 1 blps) swimming speeds (e.g., McDonald et al. 1998). Second, the high velocity treatment was discontinued during the final 1.5-month freshwater holding period because facilities were not available. Upstream migration and holding in the river prior to spawning may require considerable energy expenditure depending on the distance and geographic properties of the river being ascended (Rand and Hinch 1998). Greater effects of exercise may be realized if the exposure to high velocity environments is continued well past the termination of feeding and up to the point of reproduction. Third, females in the 'control' group (i.e., NIMP – LV) exhibited a much greater ability to deposit eggs than previous studies of captively-reared chinook salmon (D. Vindetti, IDFG, personal communication) including the Dungeness River population in the same stream channel used in the present study (Berejikian et al. 2001b). Thus, the control treatment may have provided more favorable environmental conditions than those experienced by fish in previous studies, improving their performance, and leaving little opportunity for hormone and HV treatments to improve reproductive performance as measured in this study.

Further elevating current velocities and thus forced swimming speed would likely create detectable effects (either positive or negative) on the variables measured in this study. Future studies investigating effects of exercise on maturing salmon should expose the fish to higher velocities, including the freshwater acclimation phase. Ideally, studies on exercise conditioning would be conducted in laminar flow raceways, where current velocities are relatively constant and known at nearly all points in the vessel. However, this research was initiated in attempts to improve rearing conditions in a way that could be applied to current best practices for rearing salmon captive broodstocks, which include the use of large circular vessels.

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Table 1. Definitions of behavioral frequencies, states, and durations measured from chinook salmon spawning in the stream channel.

Class	Behavior	Definition
Nesting behavior (frequency)		
	Probe	Female arches back and lowers anal fin into substrate in a developing nest
	Nest dig	Excavating dig performed during nest construction
	Cover dig	Dig which either covers recently deposited eggs or covering a previously constructed nest
Aggression (frequency)		
	To female	Total aggressive acts (threat displays + attacks) initiated by fish being observed towards other females
	From female	Total aggressive acts (threat displays + attacks) received from other females against fish being observed
	To male	Total aggressive acts (threat displays + attacks) initiated by fish being observed towardsmales
	From male	Total aggressive acts (threat displays + attacks) received from males against fish being observed
Reproductive Status (proportion)[1]		
	Courted	Attended by one or more males exhibiting courtship behavior
	Nest digging	Excavating a nest as evidenced by a deepening depression
	Guarding	Biting or chasing other fish near a previously spawned nest
Duration		
	Pre-spawning	Time between introduction to the channel and first spawning event
	Sexually active	Time between first and last spawning event
	Post-spawning	Time between last spawning event and death
	Post-release	Time between introduction to the channel and death

[1]\ Proportion of observations in which the female was at least once observed in the following 'state'

Table 2. Results of test for autocorrelated dependent variables. The Pearson correlation coefficient (r), Bonferroni-adjusted P-value (P) are shown for each pair of significantly correlated DVs. Nest digging status, courted status, and post-release lifespan were not analyzed by ANOVA. Nest dig frequency and probe frequency were combined into the variable 'nest building'

Variable 1		Variable 2			
Behavior	Class	Behavior	Class	r	P
Probes	Frequency	Nest digs	Frequency	0.671	0.005
Probes	Frequency	Courted	Status	0.745	< 0.001
Nest digs	Frequency	Courted	Status	0.619	0.030
Nest digs	Frequency	Nest digging	Status	0.821	< 0.001
Cover digs	Frequency	Courted	Status	0.888	< 0.001
Nest digging	Status	Courted	Status	0.703	0.001
Pre-spawning	Duration	Post-release lifespan (r)	Duration	0.911	< 0.001

Table 3. Results of the three-way ANOVA to determine the effects of hormone, exercise and release group on reproductive behavior. There were no significant interactions among the three main effects for any of the dependent variables. Significance of Bonferroni-adjusted P-values is indicated by an asterisk.

		Hormone		Exercise		Release Group	
Class	DV	F	P	F	P	F	P
Nest construction							
	Nest building	0.052	0.822	0.128	0.724	1.075	0.310
	Nest covering	0.957	0.337	2.552	0.123	0.881	0.357
Aggression							
	To female	4.829	0.037	0.589	0.450	0.776	0.387
	From female	2.385	0.135	0.067	0.798	0.469	0.500
	To male	15.473	0.001*	0.511	0.481	4.698	0.040
	From male	1.815	0.190	4.024	0.056	1.630	0.213
Status							
	Nest guarding	13.151	0.001*	0.181	0.674	0.064	0.802
Duration							
	Pre-spawning	8.864	0.006*	0.240	0.629	2.488	0.127
	Active	0.916	0.348	0.820	0.374	0.157	0.695
	Senescence	0.282	0.600	0.861	0.363	0.877	0.358

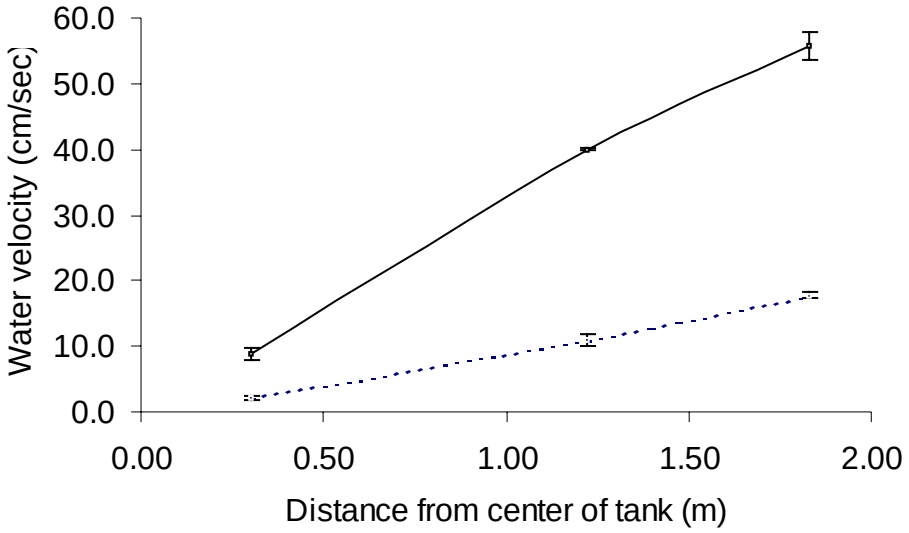

Figure 1. Current velocities in the two high velocity (solid line) and two low velocity tanks (dashed line) from 25 January through 11 July 2001. Four measurements were taken at each contour (i.e., 0.3 m, 1.2 m, and 1.8 m from center standpipe) and averaged to provide a single value at each contour for each tank. The means (±S.E.) on the graph represent the average of the two tanks.

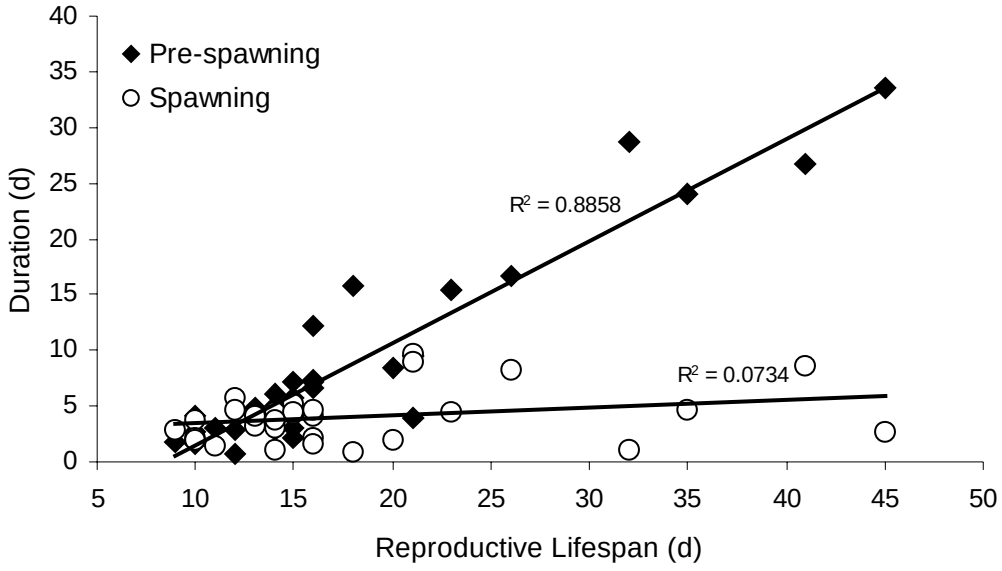

Figure 2. Relationships between (i) pre-spawning duration (time from introduction to the channel to first spawning event), (ii) spawning duration (time between first and last spawning event) and reproductive lifespan for all females.

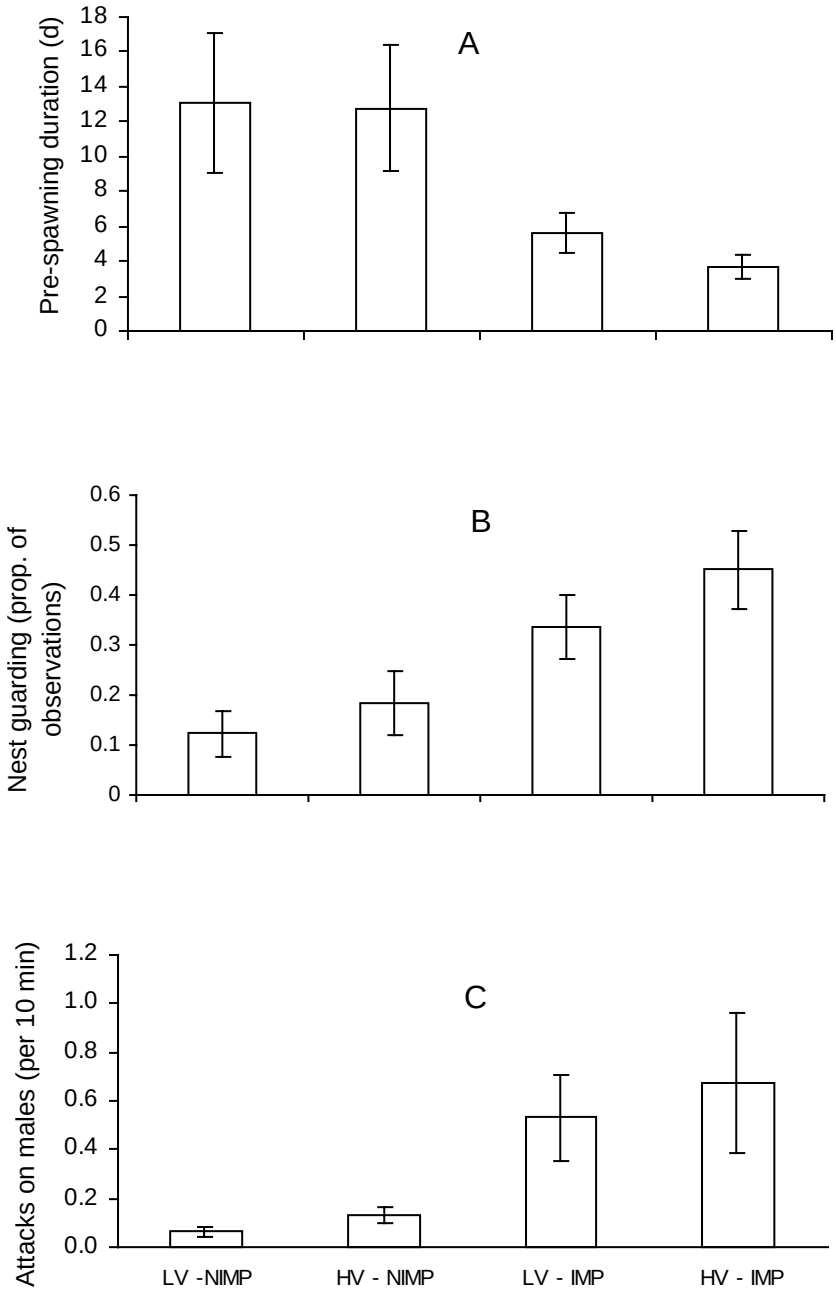

Figure 3. Reproductive behaviors significantly affected by the main effect of GnRHa implants. Graphs show cell means (± SE, n = 8) for both levels of GnRHa (implanted IMP, and non-implanted NIMP) and both levels of current velocity (high velocity HV, and low velocity, LV).

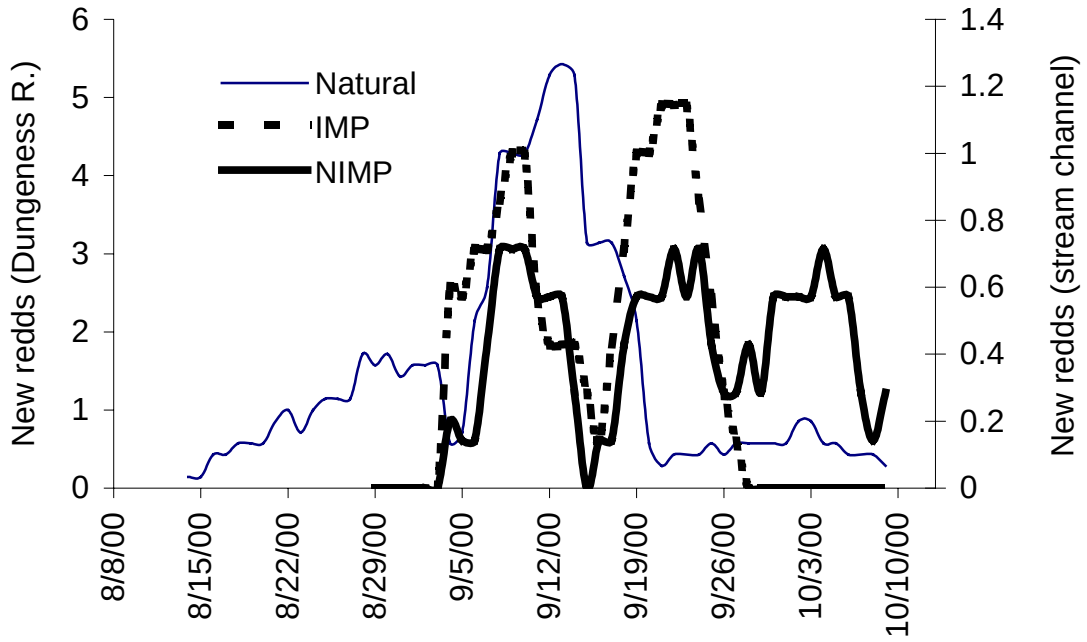

Figure 4. Temporal pattern of new redd construction by (i) wild and returning hatchery reared fish in the Dungeness River (natural), and (ii) implanted (IMP) and non-implanted (NIMP) captively-reared fish in the stream channel. Captively-reared fish were released into the stream channel on 2 September (16 fish) and 15 September (16 fish) 2000. Data from the Dungeness River are from (Marlowe et al. 2001). Lines represent a 7-day moving average**.** A 'new redd' in the spawning channel was equivalent to the construction of a female's first nest.

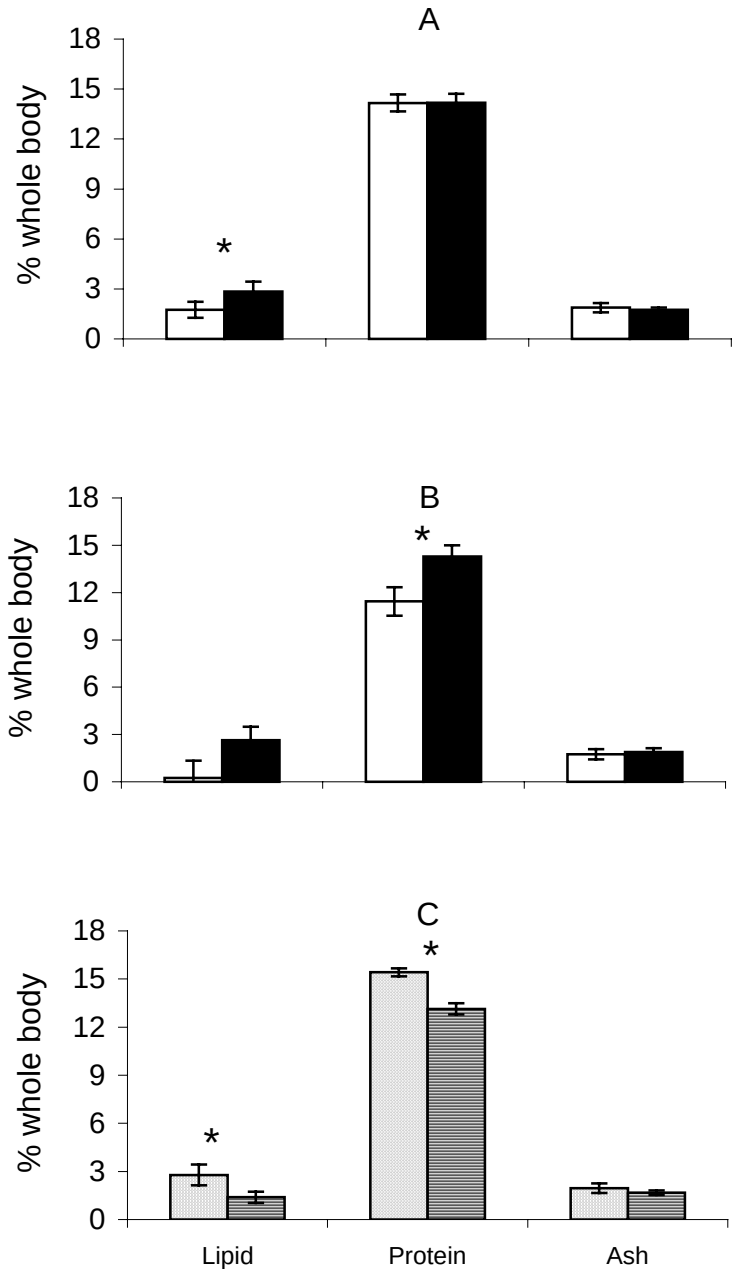

Figure 5. Mean (± SD) percentage of whole body (less gonads) lipid, protein, and ash for (A) females reared in high velocity (white bars) vs low velocity (black bars), (B) males reared in high velocity tanks (white bars) vs low velocity tanks (black bars), and (C) females spawned artificially (stippled bars) or spawned naturally in the stream channel (striped bars). Statistical significance is indicated by an asterisk ($P < 0.05$)

TASK 5. MEASURE THE BREEDING SUCCESS OF CAPTIVELY-REARED STOCKS IN DIFFERENT FACILITIES

(FINAL REPORT: 1 JUNE 2000 THROUGH 31 MAY 2001)

by

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Introduction

As part of the captive rearing strategy by the Idaho Department of Fish and Game (IDFG) for Lemhi River chinook salmon, captively reared adults are released into streams to spawn naturally. In 1998, the first releases were made of an ESA-listed stock of chinook salmon which had been reared to adults in captivity. Preliminary information from IDFG (D. Vindetti and P. Hassemer, IDFG, personal communication) indicated that their ability to spawn successfully was less than that of their wild cohorts. As captive rearing technologies improve, it is important to monitor the breeding performance of captively reared chinook salmon to allow for improvements in rearing technology. Information gathered during the monitoring efforts is used to design controlled reproductive success experiments under the Captive Broodstock Research Project (93-056-00).

Work Completed

In 2000, scientists of the National Marine Fisheries Service (NMFS) assisted the IDFG in monitoring the success of captively reared chinook salmon released into Big Springs Creek, Idaho. Big Springs Creek flows into the Lemhi River just north of Leadore, ID. In July 2000, maturing chinook salmon were measured, weighed, and identified by PIT tag code as part of the ongoing IDFG pre-release sampling program. Adults released into Big Springs Creek during mid-July 2000 included 15 BY95 fish (all females), 36 BY96 fish (32 females and 4 males), and 20 BY97 fish (3 females and 17 males). A breach in the downstream weir allowed an unknown number to escape the

study section and enter the Lemhi River. However, the weir was repaired and the majority of the remaining fish in Big Springs Creek for the duration of the study.

The behavior of fish in the stream was monitored daily by IDFG personnel between mid-July and early September. Between 10 and 16 September 2000, NMFS assisted IDFG in scanning the entire stream section available to the chinook salmon once or twice daily. During each scan observers walked the study section of Big Springs Creek. An attempt was made to locate as many fish as possible. On alternate days the upper Lemhi River was also scanned. When a fish was located its "status" was recorded. Each fish observed was assigned a specific status, as follows.

Females:

Test digging - digging in a broad area, non-focused, and indicative of exploratory digging for a nest site (Berejikian et al. 2001);
Nest digging - focused digging as part of nest construction;
Cover digging - placement of gravel on top of a redd in which eggs have been deposited;
Holding away - swimming in main current, holding position, not under cover;
Holding near redd (male present) - female near a redd or excavated area with a male nearby;
Holding near redd (male absent) - female near a redd or excavated area with no male nearby;
Inactive - relatively motionless, usually along the stream margin or under cover.

Males:

Courting - male attending a female and exhibiting courtship behaviors including crossovers and quivers (Tautz and Groot 1975; Schroder 1981; Berejikian et al. 1997);
Satellite - male closely associated with the courting pair but positioned slightly downstream. Satellites had frequent aggressive interactions, but were subordinate to the dominant (courting) male;
Holding near female - male positioned close to female but exhibiting no courtship behavior;
Wandering - actively swimming or holding position in fast currents, but not competing for access to or courting a sexually active female.
Inactive - same as for females.

To eliminate duplicate reporting of data, IDFG and NMFS collaborators agreed that all data collected were to be summarized for the purposes of producing a final report by IDFG. Therefore data recorded by NMFS personnel from the above work were communicated to IDFG technicians in the field on 15 September 2000. NMFS will continue to provide technical support during data analysis and report as requested by IDFG. For example, in 2001 the effects of temperature prior to final maturation will be tested for effects on the onset of reproduction. This task will be continued in future years to determine improvements (or not) in the breeding success of captively reared chinook salmon as changes to culture technologies are made. Information gathered from these studies will continue to be shared between IDFG and NMFS through the Snake River

Chinook Salmon Captive Propagation Technical Oversight Committee process. Results will help to guide future research under this project as it relates to quality of adult salmon.

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TASK 6. THE EFFECTS OF GROWTH RATE ON INCIDENCE OF EARLY MATURITY AND ADULT QUALITY IN SPRING CHINOOK SALMON

(PROGRESS REPORT: 1 JUNE 2000 THROUGH 31 MAY 2001)

by

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Introduction

One critical problem for captive rearing of chinook salmon is the loss of fish due to early sexual maturation of males. In many species males may mature early relative to females, with the incidence varying among species, stocks, and rearing conditions for cultured fish. In a captive broodstock program it is undesirable to produce mature males at a time when females of the same stock are not mature. For example, current release strategies for recovery of Snake River chinook salmon in particular include release of mature adult fish, and in the captive broodstock programs which spawn fish artificially cryo-preservation has not yielded consistent quality milt. In addition selective mortality of precocious males could reduce the effective breeding population size (N_e) of captive broodstock. This problem is being encountered in several of the Snake River chinook salmon stocks presently in captive broodstock programs. Early maturation of males as high as 60% has been observed in some stocks. Thus there is a critical need to develop methods to control age of maturity and minimize asynchronous maturation of males and females in captive broodstock programs for endangered fish species.

Research to date, primarily from Atlantic and chinook salmon indicates that genetic, biotic, and abiotic factors influence the incidence of early male maturation (Rowe and Thorpe 1990a, 1990b, Rowe et al. 1991). The relative importance of these

factors and how they interact are poorly understood. Because genetic selection should be minimized in a captive broodstock program for depleted stocks, rearing strategies that minimize expression of the trait are needed. Additionally, strategies which alter the seasonal timing of maturation must be avoided. It may be possible to reduce the incidence of early male maturation through alteration of abiotic conditions such as water temperature and photoperiod, or biotic factors such as growth rates or diet composition. The approach is to identify factors systematically which influence maturation and seasonal periods when maturation is initiated, and then use this information to develop diets and growth regimes and rearing strategies which reduce the incidence of early male maturity. Diets and growth regimes are being developed which sustain growth and provide sufficient stored energy for appropriate life-cycle transitions, development of gametes in adult fish, and achieve target size for release as adult fish.

For development of a diet, growth regimes and rearing strategies that minimize early maturation of male spring chinook salmon, the research program is following two key areas of investigation. First, develop diets and feeding regimes to modify body composition and growth, and second, define critical periods when initiation of maturation is influenced by metabolic factors. In initial studies, a significant positive correlation of the percent of males maturing at 2 years of age with body fat levels was found (Shearer and Swanson 2000). Later, in an experiment in which both size and body fat levels were manipulated, it was found that growth rate or size one year prior to maturation was the primary factor affecting maturation at 2 years of age.

During the previous phase of the project, one study attempted to determine the threshold of size or growth rate above which male maturation is initiated. Fish were fed high protein, low fat diets of graded ration levels. Fish size during the first autumn ranged from 50 - 100 g. In that study, 65 - 90% of the males matured 1 year later. Therefore it was concluded that even though size or growth rate clearly influenced the rate of male maturation, the threshold had been exceeded. In the present work plan we propose to rear fish on graded rations of a commercially available diet and target a much lower body size for the first year of rearing than that of the previous study. Based on previous studies and data from wild fish it is clear that, if the threshold of size exists, it is probably below 20 g and occurs in December of the first year of rearing. Therefore, the present study was designed to determine a threshold of size for initiation of maturation in male spring chinook salmon.

Methods

Experimental design and fish

The hypothesis being tested in this experiment is that a threshold growth rate (or size) exists for the initiation of male maturation, and that this threshold is achieved during the autumn/winter 1 year prior to maturation. Eggs from Willamette River spring chinook were incubated at 5 °C to delay hatching and emergence as long as possible. The fry were ponded into 5 °C recirculated fresh water at the Northwest Fisheries Science Center Montlake Hatchery, and were fed a commercial feed (BioOregon starter) for the first 2 months. At this time fish were split into five groups (duplicate tanks, 350

fish/tank) and fed a commercial grower diet (BioOregon) at one of five feeding levels based on predetermined growth trajectories. Water temperature was increased to 10 °C during the summer 2000. A natural photoperiod was maintained. The goal was to produce fish of 10, 15, 20, 25, and 30 g in weight on December 1, 2000. This is within the size range of naturally-reared, or wild, fish. Two additional tanks of fish were reared as controls. These fish were reared at ambient temperature and fed to satiation and their projected weight in December was 70 g.

Lipid determination

Ten fish from each tank were randomly selected for determination of weight, length and body composition approximately monthly over the first year and approximately every 6 weeks thereafter. Fish were sexed beginning with the day 269 sample. For proximate analysis, fish smaller than 1 g were pooled (10 fish) and fish between 1 and 100 g were chopped, dried, and ground in a mortar and pestle. Fish larger than 100 g were ground in a food processor and a sub sample of 100 g of wet material was dried then reground, and sub-samples were taken for analysis. Moisture was determined by drying to constant weight at 105 °C, fat was determined using a Soxhlet device with dichloromethane as the solvent. Moisture and fat analysis were performed on individual fish over 1 g (10 fish /tank).

Hormone analysis

Fish were killed in a lethal dose of MS222, weighed, and measured. Blood was collected via the caudal vein, initially using heparinized capillary tubes after severing the caudal peduncle, and then heparinized syringes once fish grew larger. Blood was centrifuged at 3,000 x g for 3 minutes and plasma stored at –70 ^{o}C. Plasma 11-ketotestosterone was measured according to Schulz (1984) using a methylene chloride extraction method described in Planas and Swanson (1994).

Statistical analysis

Tank means were used as the unit of observation. ANOVA and Fisher's PLSD test was used to conduct multiple mean comparisons. All analyses were performed using Statview^{TM} (Abacus Concepts, Berkeley, CA, 1992).

Work Completed

Growth and body composition

Mortality during the period from first feeding (February 2000) to December 2000 was less than 1%. Mean fish weights in each treatment, based on the grand means were 11.3, 16.4, 19.3, 22.1, 28.9, and 110 g on December 8, 2000 (day 342) (Figure 1). Monthly sampling (10 fish/tank) and whole body fat analysis indicate that body fat levels are positively related to ration levels (Figure 2) but increases in male fatness associated with sexual maturation that have been observed in previous studies are not yet apparent.

Hormone analysis

Plasma 11-ketotestosterone measurements were on plasma samples collected during December 2000 from male fish (30 fish/tank) in three treatments: 15, 30, and 110

g (70 g treatment, Figure 3). Levels of 11-ketotestosterone were bimodally distributed in some treatments, presumably due to the presence of maturing and nonmaturing males. Fish with levels of > 1ng/ml were classified as maturing. Using this criterion, the maturation rates in the 15, 30, and 110g groups were 0%, 32% and 80%, respectively. These data suggest that the threshold size in early December for initiation of sexual maturation in males may be between 15 and 30g.

Work to be Completed

The fish will continue to be reared until it is possible to obtain an accurate estimate of the incidence of male maturation based on gonadosomatic index and visual examination of the testis. The fish will continue to be sampled approximately every 6 weeks to determine body composition and state of sexual maturation. We expect to terminate the experiment by July 2001. Sample analyses and preparation of manuscript will be completed by January 2002.

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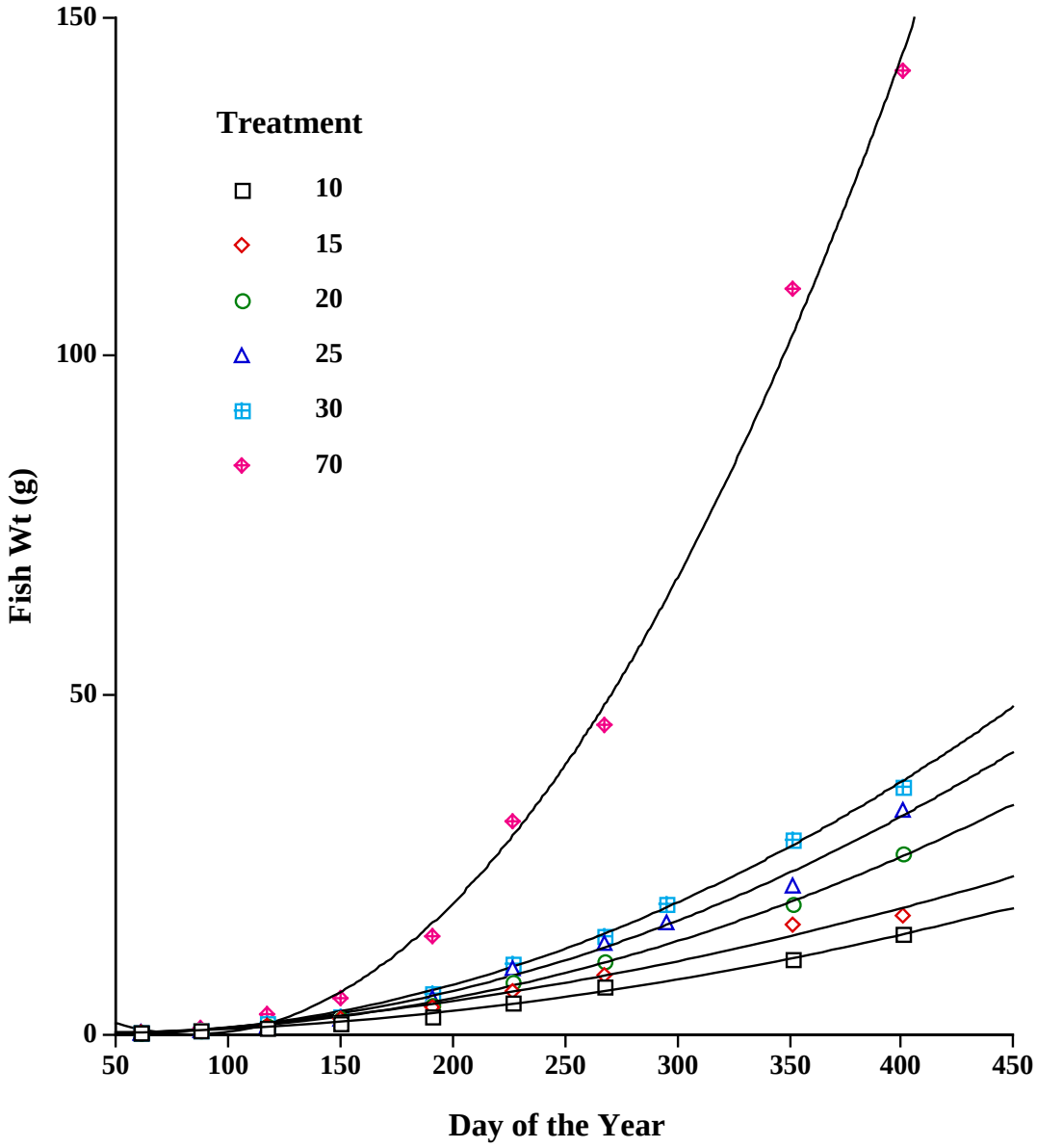

Figure 1. Growth of juvenile chinook salmon fed at various ration levels from first feeding. N = 2 tanks per treatment. Treatment is the projected weight (g).

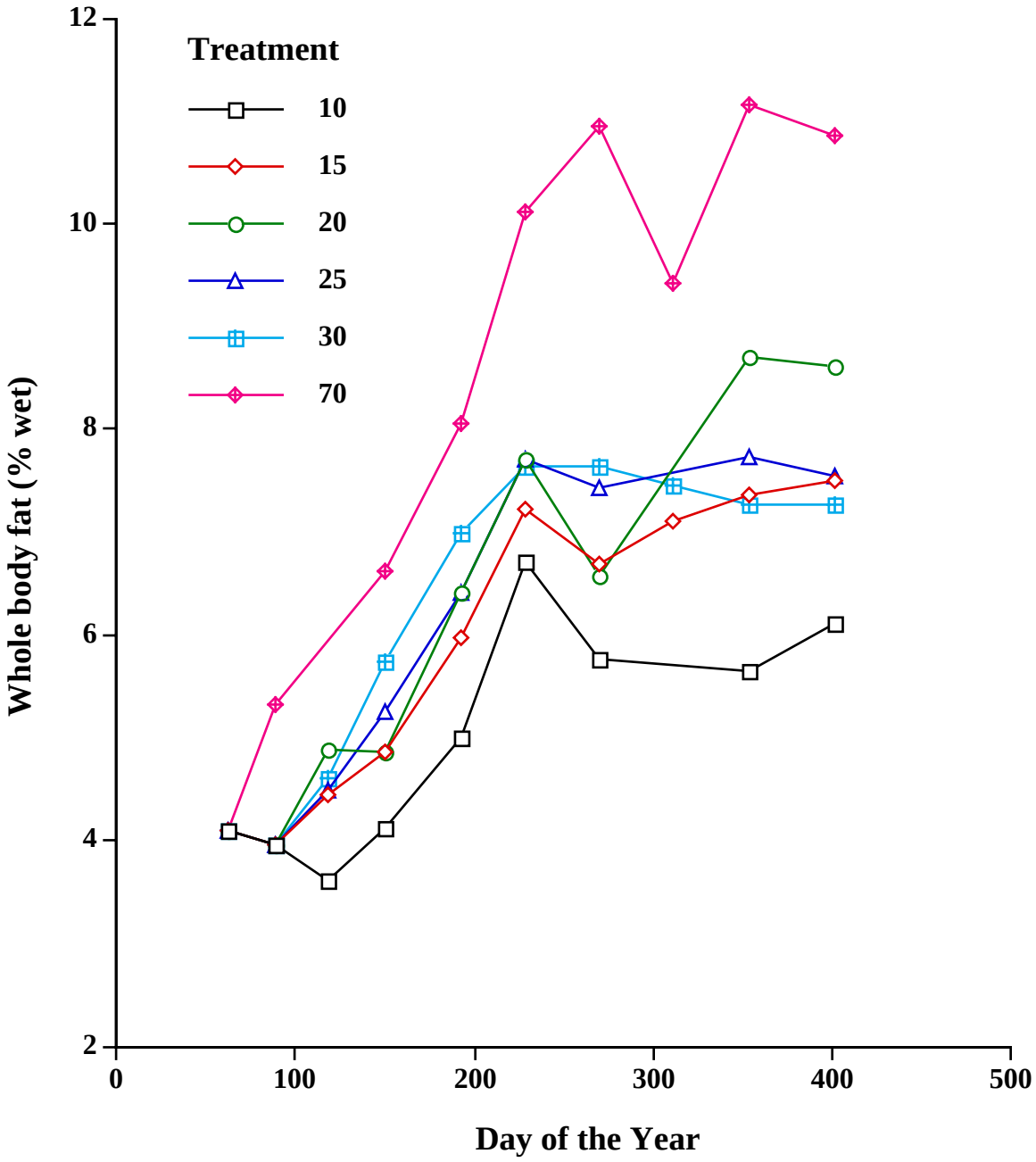

Figure 2. Whole body fat of juvenile chinook salmon fed at various ration levels from first feeding. N = 2 tanks per treatment.

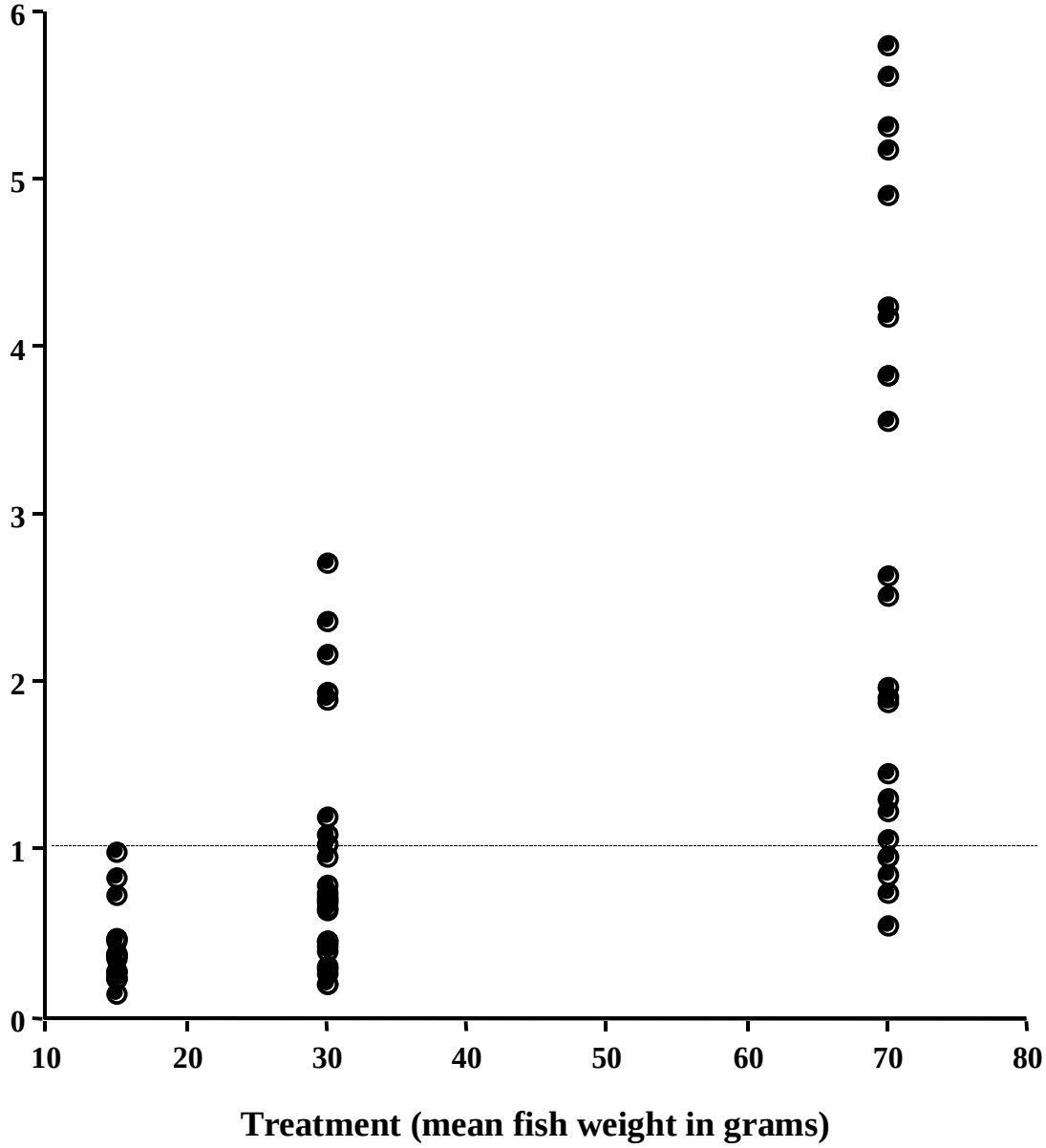

Figure 3. Plasma 11-ketotestosterone (11-KT) levels (y-axis ng/ml) of juvenile chinook salmon of approximately 15, 30, and 110 g in December (day 353). The line represents the estimated plasma 11-KT level (1.0 ng/ml) at initiation of maturation.

TASK 7. EFFECTS OF GROWTH ON AGE OF MATURATION, FECUNDITY, EGG SIZE AND EGG QUALITY IN FEMALE COHO SALMON

(PROGRESS REPORT: 1 JUNE 2000 THROUGH 31 MAY 2001)

by

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Introduction

One of the major aims of captive broodstock programs for recovery of depleted stocks of Pacific salmon is to optimize the production of fertile gametes from adult fish which will either be introduced to the wild or spawned artificially in captivity. However, various forms of reproductive dysfunction have been observed which limit the desired production from captively-reared fish and drastically reduces the effectiveness of such programs for recovery of depleted stocks.

Both the Redfish Lake sockeye salmon and Snake River spring chinook salmon captive broodstock programs have encountered problems with highly variable egg quality (range: 0 - 90%, and averages of 40 - 70% survival to eyed-stage). This is much lower than that of populations of anadromous fish spawned artificially on return to the hatchery. In addition, the seasonal timing of ovulation in some of the captive populations has been 3 - 5 weeks later than wild counterparts (P. Kline, Idaho Department of Fish and Game, personal communication), making spawning of captively-reared adults with wild fish improbable. Captively-reared females also display a high rate of egg retention and, in

some cases, abnormal ovarian development leading to reduced egg size and number, and atretic eggs.

Problems of reduced fertility and delayed maturation may be due to effects of rearing environment on ovarian development. In farmed rainbow trout one of the leading causes of poor egg quality is inappropriate timing of egg collection relative to ovulation. Therefore the seasonal delay in ovulation and asynchronous maturation of oocytes within the ovary may be a manifestation of abnormalities in the rate of oocyte development due to inappropriate environmental cues, such as temperature and food availability. Abnormal ovarian development may be due to rearing conditions early in the life cycle when physiological commitments to egg number are occurring, and/or during the final year prior to spawning when secondary oocyte growth is occurring. Essential to developing solutions to this problem is understanding what non-genetic factors determine the number and size of oocytes that reach final stages of maturation and ovulation, and what factors regulate the growth and maturation of the oocyte.

Research on other fish species indicates that ration, growth rate, and/or body size during critical periods of the life cycle can affect the age of maturity (initiation of secondary oocyte growth), maintenance of ovarian growth and the degree of ovarian growth. For example, in rainbow trout a reduction in ration 9 - 12 months before maturation reduced the number of fish which matured that year, while similar reductions in ration 4 - 8 months prior to ovulation reduced fecundity but did not alter the number of fish maturing (Bromage et al. 1992). When body weight was increased near the end of the cycle (4 months prior to spawning), there was no change in egg number or size; however, there was a decrease in relative fecundity (fecundity/body weight). In other studies in salmon, growth during the initial freshwater stages correlated to final egg size and/or fecundity (e.g., Jonsson et al., 1996; Morita and Takashima, 1998; Jonsson and Jonsson 1999). Body size just after smoltification has been related to the decision to mature the following year (see Duston and Saunders 1999).

In semelparous species the response to factors which influence the physiological commitment of oocytes to incorporate yolk and mature is crucial, as there is no second opportunity once this commitment is made. In Pacific salmon little is known about critical periods in ovarian development whereby growth can alter the egg number, egg size, or egg quality. In general, hatchery managers focus on feeding high quality broodstock diets during the year of expected maturity and do not consider the effect of juvenile rearing strategies and diets on the reproductive performance of adults 1 - 3 years later. This factor has become increasingly important because of continued problems with egg quality in captive broodstock programs, where the goals are to produce large numbers of viable gametes and offspring genetically representative of the remaining population. The prevention of the loss of potential eggs to atresia (cell death) during ovarian development can be crucial to the success of a recovery program. The manipulation of ration to control maturation also has important implications for optimizing egg production in limited water supplies, stocking density, and feed costs.

The tools are now available to investigate the mechanisms whereby growth during various points in the life cycle can impact reproductive performance. Previous work suggests that seasonally dynamic patterns of growth are important for regulating smoltification (Beckman et al. 1998) and it is probable that this may also be an important environmental cue for timing of commitments to gonadal versus somatic growth. In this study, the effects of growth during the smolt to adult period on egg size, relative fecundity and egg fertility of coho salmon were examined. Coho salmon have been selected for this study because of the simple life history, and the availability of fish from a previous study which examined the effects of growth rate on smoltification.

Methods

The experimental approach is to produce female coho salmon of a range of body sizes and growth rates by varying ration and to determine the relationship between body growth during various points of the life cycle and ovarian development. Yearling coho salmon (1997 brood) were obtained from Minter Creek Hatchery, WA and reared in 12-ft diameter fiberglass tanks supplied with filtered and UV-treated seawater at the NMFS Manchester Research Station. Fish were individually tagged with PIT tags prior to transfer to seawater as smolts during spring 1999. Fish (1,300) were divided into two groups and reared on either high (0.8% body weight/day) or low (0.5% body weight per day) ration through September 1999. Periodic sampling for growth, body composition, and plasma hormone levels were made regularly during 1999 as part of another study on smoltification (B. Beckman, NWFSC, personal communication). From September 1999 to January 2000 the remaining 800 fish were reared on constant ration, and body weights and lengths were recorded monthly. In October 1999, fin tissue was collected from all fish for determination of genetic sex using a molecular marker for the Y-chromosome (Du et al. 1993). This marker has been validated for use in three Puget Sound stocks of coho salmon. Because of limited tank space and seawater, most of the males were culled from the experiment. In January 2000, fish were sorted by size into two groups: those above the median size (large) and those below the median (small). These two groups were further subdivided into two tanks of high (0.8% body weight/day) and low (0.5% body weight per day) ration. Thus, there were four treatments (70 females, 10 males per treatment), one tank per treatment: Big High Ration (BH), Big Low Ration (BL), Small High Ration (SH) and Small Low Ration (SL). Fish were fed a commercially available diet (Moore Clark, BroodSelect) until 6 November 6 2000 when food was withdrawn and the fish were transferred to freshwater tanks. From 6 November through 27 December 2000, fish were checked every 4 - 7 days for ovulation.

Body weight and length were recorded bimonthly for all fish from January 2000 through July 2000, and during October 2000, to monitor growth during the adult rearing period. At spawning, data on body weight (before and after removing ovulated eggs), body length, total egg mass, number of ovulated eggs, individual egg mass, and spawning date were recorded. Similar data were obtained from adult hatchery (adipose fin-clipped) and wild (non-adipose fin-clipped) fish captured in the weir in Minter Creek and returned to the Minter Creek Hatchery. For females that did not mature at 3 years of age, data on body length and weight, and ovarian weight were recorded in January 2001. All fish

were killed at this point and samples of ovaries of the nonmaturing fish were fixed in Bouin's fixative for 24 hr and then stored in 70% ethanol for subsequent histological analysis.

Data was analyzed by one-way ANOVA or by multiple step-wise regression using Staview (Abacus Inc., CA).

Results

Analysis of data collected during the experimental period is ongoing and expected to be completed by November 2001. The initial results are described below.

Growth and maturation

Individual growth profiles for the experimental period (Figure 1) and spring-summer 2000 (Figure 2) indicated that the greatest increases in body weight occurred from July to October 2000. At age 3, 52% (85 out of 137) of the females matured and were spawned during November and December 2000. The remaining 38% (52 out of 137) of the females that did not mature at age 3 could be classified into two groups based on gonadosomatic indices (GSI) in January 2001. Approximately 44% (23 out of 52) of the nonmaturing fish had GSIs > 0.5, and 56% (29 out of 52) had GSIs < 0.3. Although histological analysis of the ovaries from the nonmaturing fish has not been completed, gross examination of the ovaries indicated that fish with GSIs < 0.3 had abnormal oocytes and resorbed eggs. On the other hand, fish with GSIs > 0.5 had normal appearing oocytes in early stages of vitellogenesis. Therefore, all data on growth were split into three groups: maturing females, nonmaturing females with large developing ovaries (NM-LO), and nonmaturing females with small, abnormal ovaries (NM-SO).

The pattern of body weight changes (Figure 3) during the period of rearing of these three groups indicated that there were differences in growth patterns. Maturing fish were significantly larger than nonmaturing fish with normal ovaries from October 1999 through spawning. Body weight in nonmaturing fish with abnormal ovaries was similar to maturing fish from April 1999 through February 2000; however, from May 2000 through the end of the experiment body weight of the nonmaturing fish with abnormal ovaries was significantly lower than fish that matured in December 2000. By January 2001 there was no difference in final weight between the two groups of nonmaturing fish; however, the pattern of growth over time in these two groups differed considerably. Fish with abnormal small ovaries at the end of the experiment had higher growth rate than nonmaturing fish with normal ovaries from June 1999 through February 2000. From that time (February) through October 2000, nonmaturing fish with abnormal ovaries had significantly lower growth rate than nonmaturing fish with normal ovaries, except for the period from May-July 2000 period.

Growth and egg size versus egg number

The final stripped body weight in maturing fish was significantly correlated with total egg mass ($P < 0.05$, Figure 4), individual egg weight (Figure 5), and fecundity (Figure 6); however, the R^2 for the later is only 0.27. This suggests that other factors

than body size may co-determine individual egg size. Step-wise regression analysis revealed that growth during the summer previous to spawning had the largest influence on final body weight (Table 1). Size during July 2000 was highly correlated with final body size (R^2= .083), followed by size during May (R^2= 0.49). Body size during the year previous to spawning (August 1999-February 2000) could account for only about 20% of the variance in final body size.

The relationship between body weight and either total egg mass or fecundity fell into four major stanzas (Table 2)

(i) Summer 1999: From April through June 1999, when body weights were poorly correlated with egg mass or fecundity ($R2 \leq 0.05$).

(ii) Fall to winter: From August 1999 through February 2000, when about 20% of the variance in total egg mass and 10% of variance in fecundity could be explained by body weight during that time.

(iii) Spring 2000: Body weight during May 2000 explained about 50% of the variance in total egg mass and 30% of the variance in fecundity.

(iv) Summer to fall 2001: When body weights during July and October were highly correlated with total egg mass (R^2= 0.81-0.94) and fecundity (R^2= 0.64-0.66).

Individual egg mass was poorly correlated with body size. In the regression analysis the highest correlation of individual egg size with body weight was found during October (R^2= 0.27). Regression analysis of the residuals of individual egg mass versus strip body weight and fecundity versus strip body weight (Figure 7) indicated that size influenced the relationship between egg number and egg size. Small fish tended to produce large number of small eggs compared with large fish which produced small numbers of large eggs.

The mean body weight of the Manchester-reared Minter Creek stock of coho salmon used in this study was generally smaller and had a narrower range than that of wild or hatchery fish that were captured in the Minter Creek Weir and spawned at the Minter Creek Hatchery in December 2001. However, the relationships between body size at spawning and total ovary mass (Figure 8) and fecundity (Figure 9) were similar among these three groups of fish. Unlike the experimental fish reared at Manchester, more than half of the variation in individual egg mass (Figure 10) in the hatchery fish could be explained by body size at spawning (R^2 = 0.61). This may be due to the wider range of body size of the hatchery fish compared with the experimental fish because the range of data points influences the regression analysis.

Preliminary Conclusions

Studies in both Atlantic and Pacific salmon have suggested that there are two critical periods during which growth affects age of maturity in males. First, there is a period in the fall one year prior to spawning when maturation is initiated in response to high growth. Second, there is a critical period in the spring just prior to spawning when high growth permits maturation to continue. In the present study, the data support the idea of an initiation period in the fall and permissive period in the following spring. The pattern of growth during the 19 months of rearing affected age of maturity in female coho

salmon. Fish that matured in December 2000 at age 3 showed significantly higher growth during the previous fall (September 1999 onward) than nonmaturing fish. Among the nonmaturing fish, those with atretic ovaries at the end of the experiment had high growth in the previous fall like the maturing fish, but growth in the following spring was reduced. These data suggest that maturation was initiated but not sustained in the following year and atresia ensued. The nonmaturing fish with what appeared to be normal immature ovaries at age 3 would be expected to mature at age 4. These fish had comparatively low growth in both the fall and spring periods.

Growth during the summer and early fall just prior to spawning was the strongest determinant of final body size, total ovary mass, and fecundity. Less than 20% of the variance in individual egg size could be explained by body size, suggesting factors other than growth in the adult phase of the life cycle influence egg size.

Work to be Completed

Histological analysis of ovaries of immature fish will be completed by September 2001. Completion of data analysis and fecundity estimates of immature fish and preparation of manuscript for publication is expected by November 2001.

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Table 1. Regression coefficient (r^2) for the relation between stripped body weight (at spawning in December 2000), and length or weight at a given date.

Month	Weight	Length
October 2000	0.98	0.92
July 2000	0.83	0.79
May 2000	0.49	0.43
February 2000	0.22	0.21
December 1999	0.23	0.18
November 1999	0.2	0.17
September 1999	0.22	0.23
August 1999	0.19	0.17
June 1999	0.07	0.05
April 1999	0.009	0.008

Table 2. Regression coefficients (r^2) for the relationship between weight at a given date and total egg mass, individual egg size, and fecundity at spawning. Fish were spawned in December 2000

Month	Total egg mass (g)	Individual egg size (g)	Fecundity
October 2000	0.94	0.27	0.66
July 2000	0.81	0.18	0.64
May 2000	0.5	0.13	0.33
February 2000	0.2	0.06	0.1
December 1999	0.2	0.06	0.1
November 1999	0.16	0.05	0.08
September 1999	0.19	0.07	0.07
August 1999	0.18	ns	0.12
June 1999	0.05	ns	0.05
April 1999	ns[1]	ns	ns

1/ ns = no sample

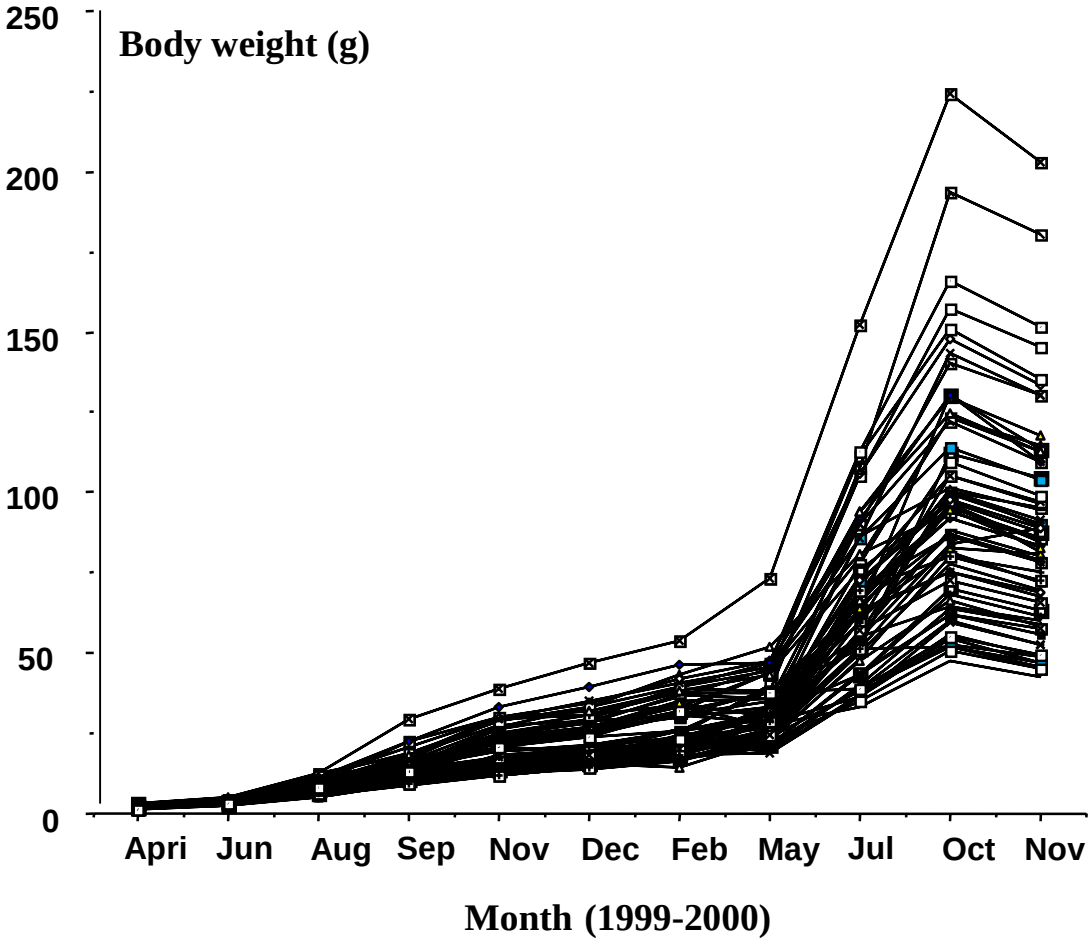

Figure 1. Body weights of individually-tagged female coho salmon during the experimental period (April 1999-December 2000).

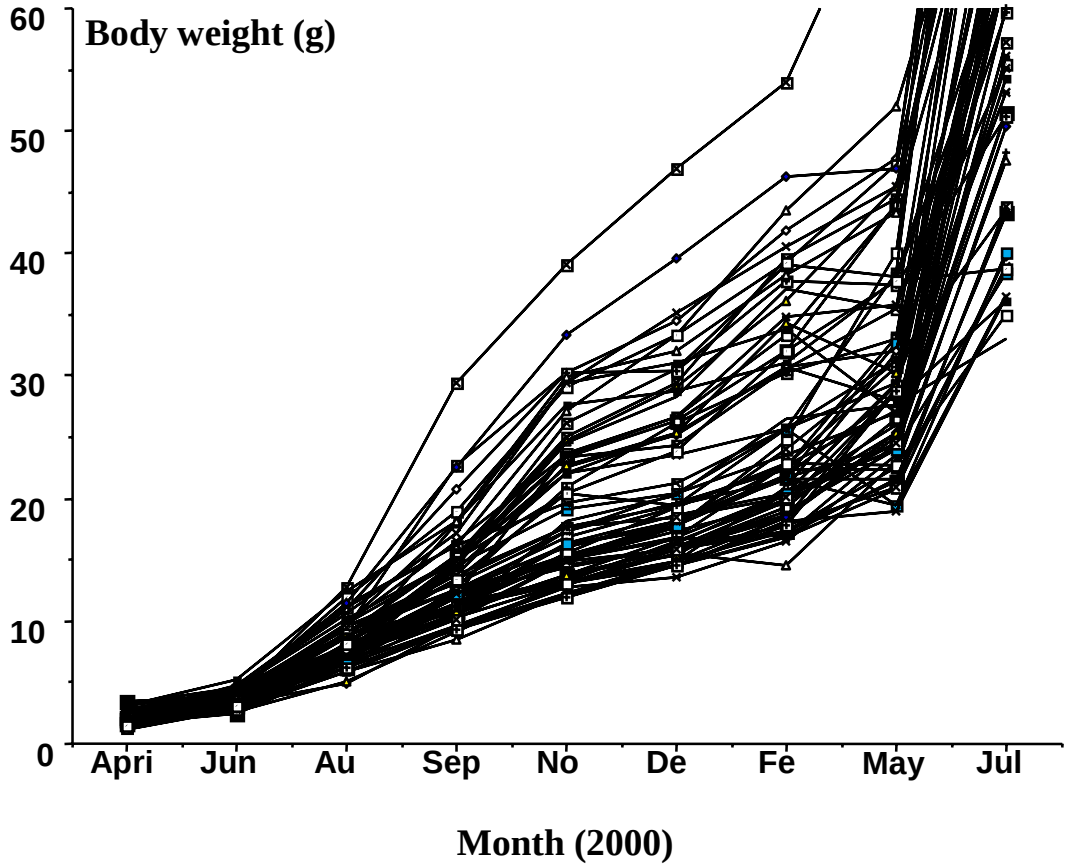

Figure 2. Body weights of individually tagged female coho salmon during the first year of rearing in seawater (April 1999-May 2000).

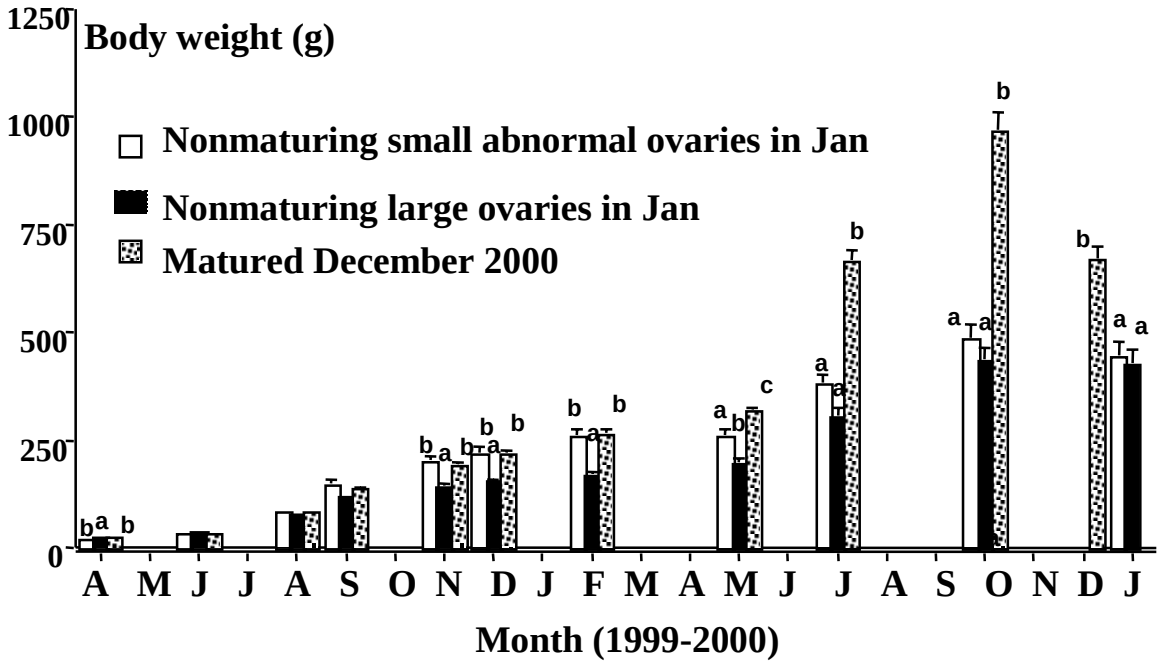

Figure 3. Mean body weights of maturing and nonmaturing female coho salmon during the experimental period. Nonmaturing fish were separated into two groups based on GSI: one with small atretic ovaries (GSI < 0.3) and the other normal immature ovaries (GSI > 0.5). Maturing 3-year old fish were spawned during December 2000. Gonads were collected from nonmaturing fish in January 2001. Same letters above bars indicate non-significant differences within a sampling period.

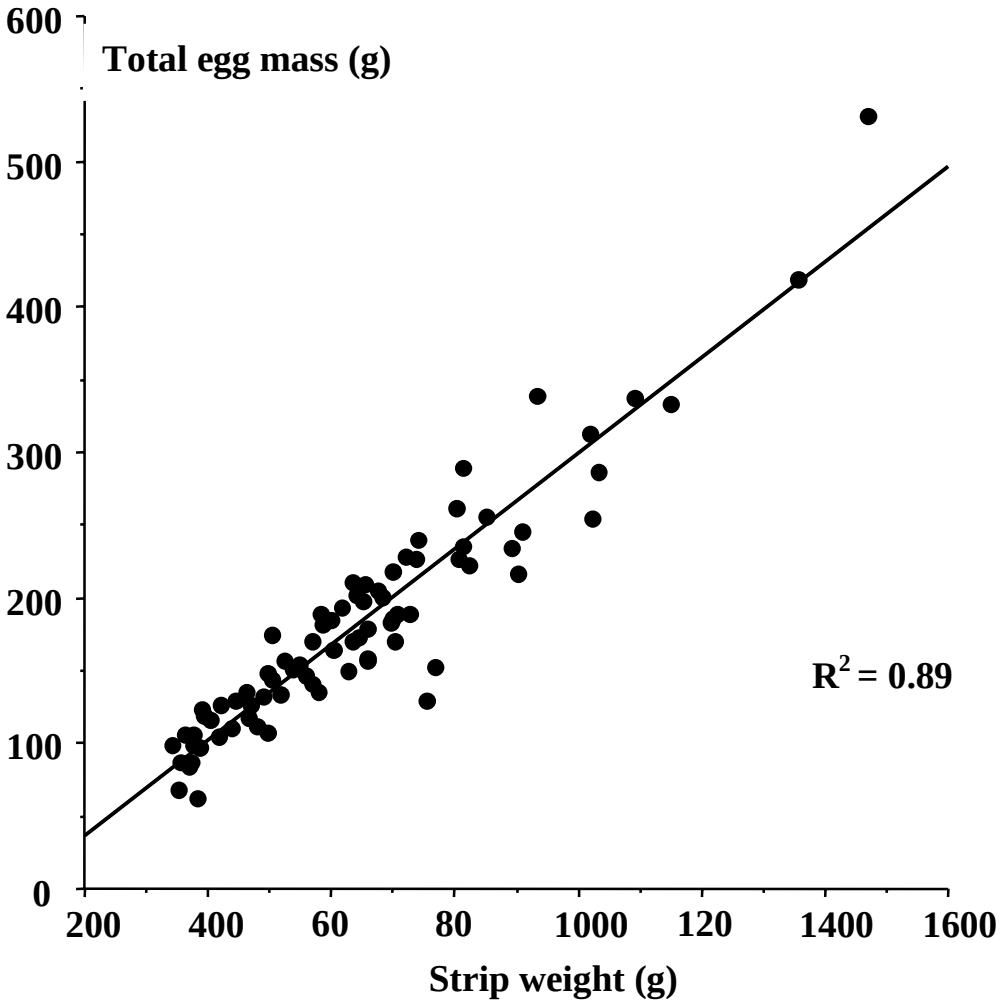

Figure 4. Regression analysis of total ovary mass and strip body weight at spawning.

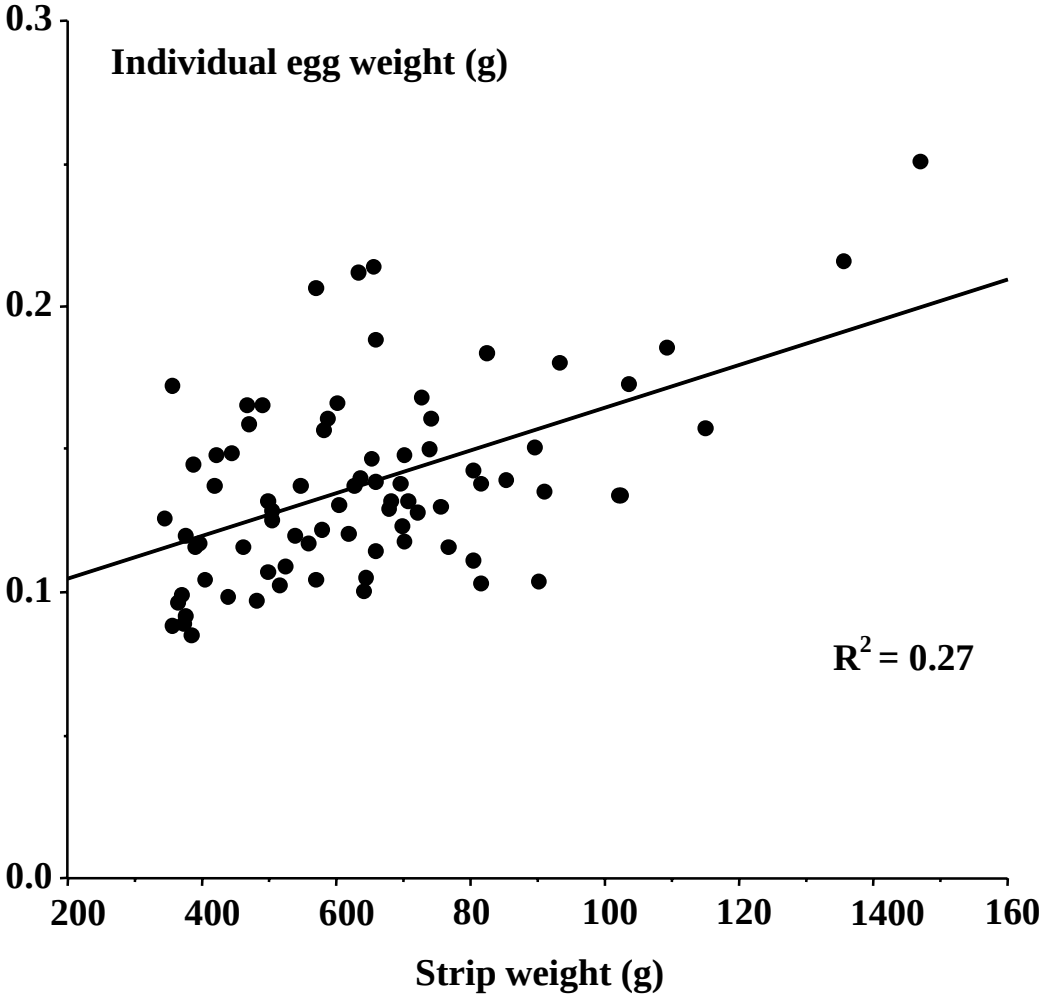

Figure 5. Regression analysis of individual egg mass and strip body weight at spawning.

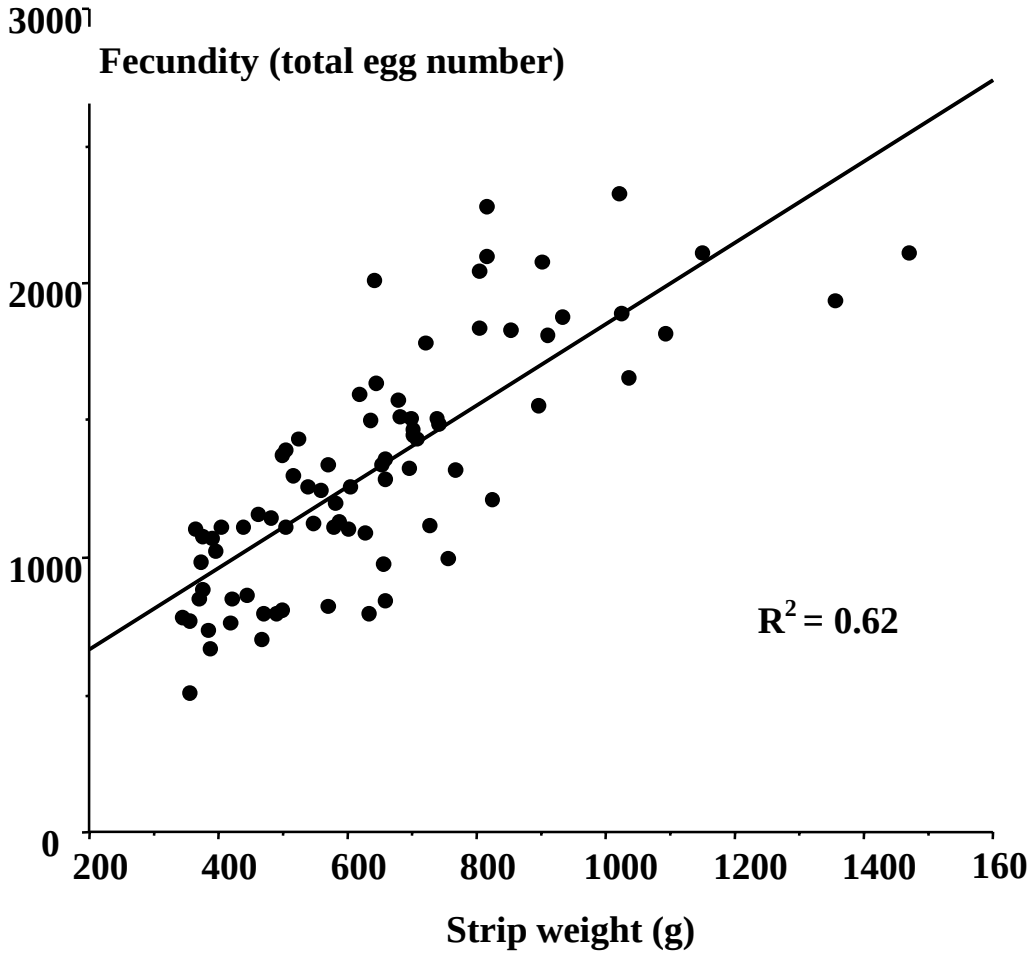

Figure 6. Regression analysis of fecundity and strip body weight at spawning.

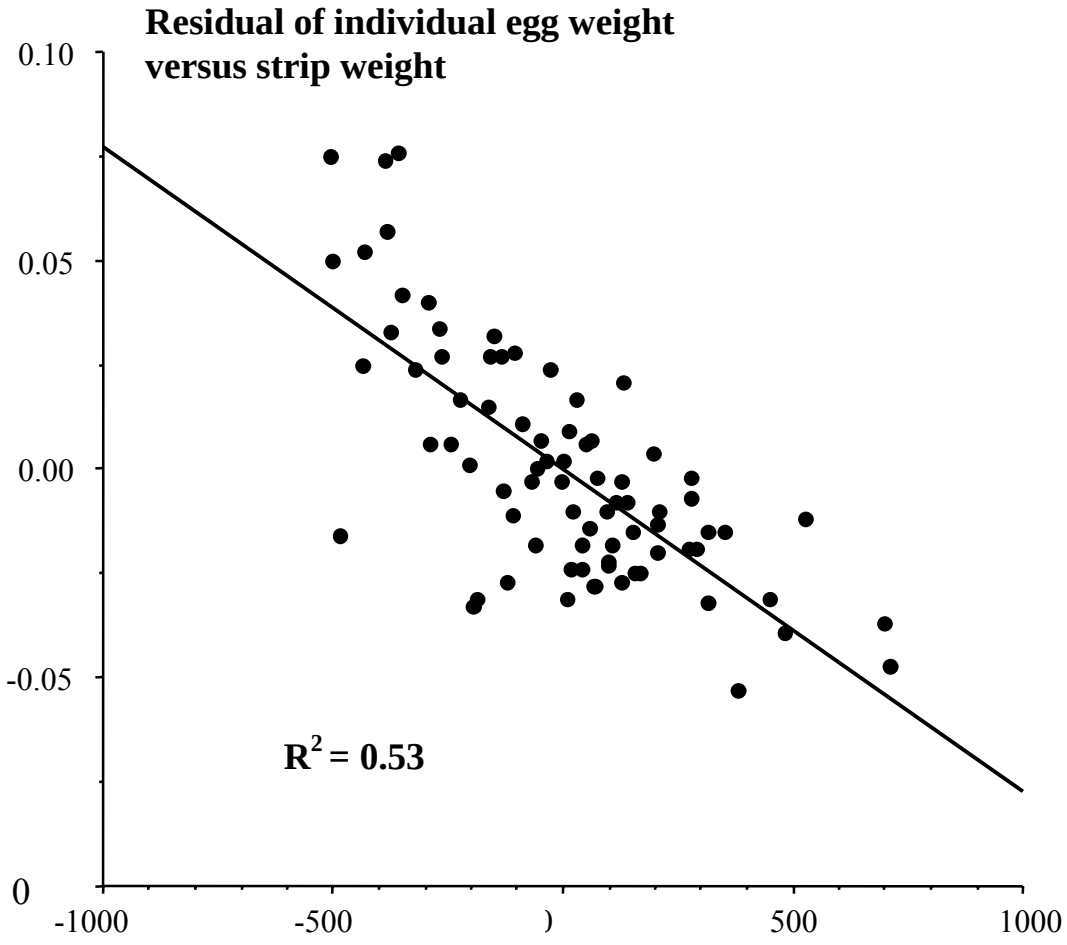

Figure 7. Regression analysis of the residuals of individual egg mass versus strip body weight and fecundity versus strip body weight.

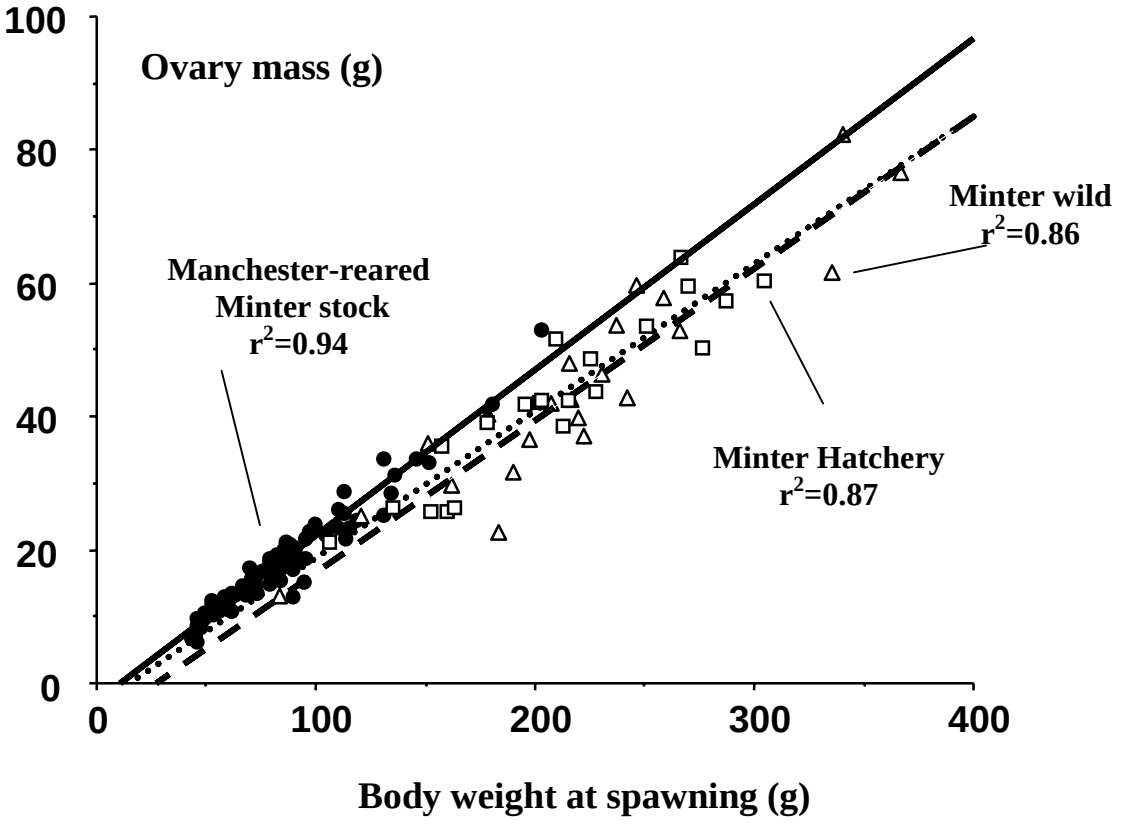

Figure 8. Total ovary mass versus strip body weight for Minter Creek coho salmon reared at Manchester, Minter Creek Hatchery, and wild adult coho salmon captured in the Minter Creek weir during November and December 2000, and spawned in December 2000.

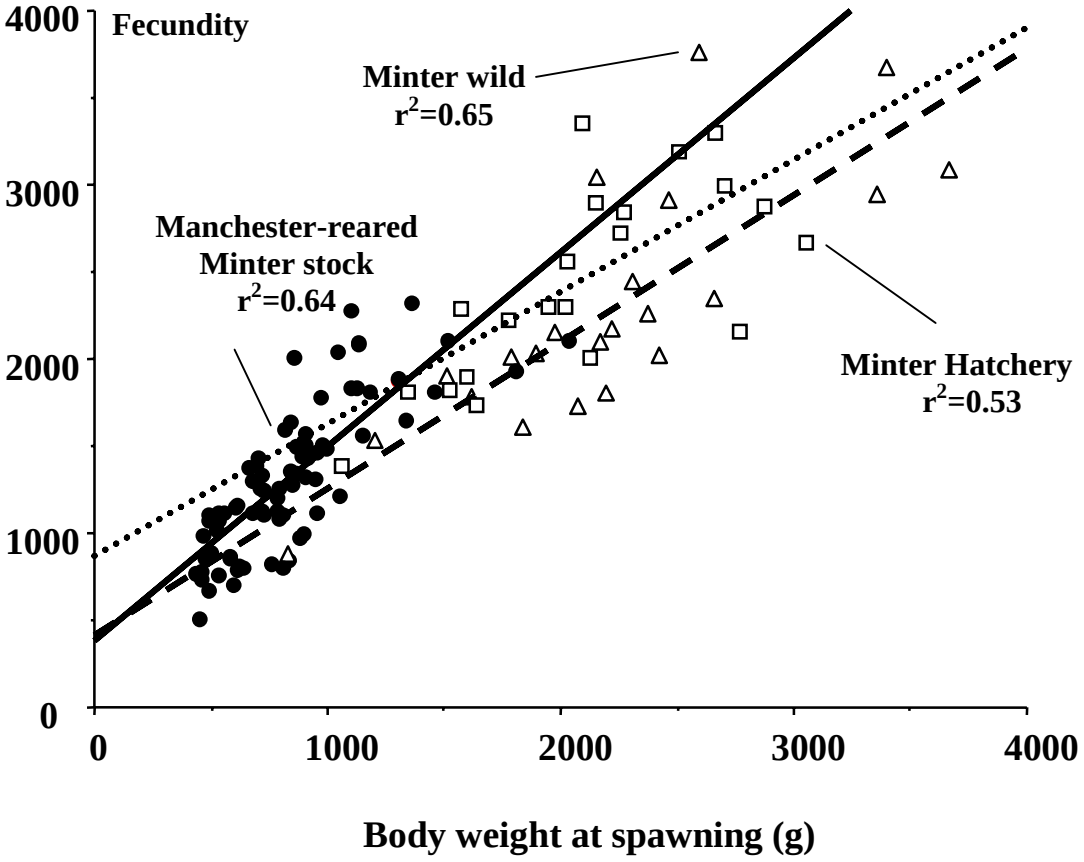

Figure 9. Fecundity versus strip body weight for Minter Creek coho salmon reared at Manchester, Minter Creek Hatchery, and wild adult coho salmon captured in the Minter Creek weir during November and December 2000, and spawned in December 2000.

TASK 8. INFRASTRUCTURE DEVELOPMENT AT THE MANCHESTER RESEARCH STATION FOR FUTURE RESEARCH ON ADULT QUALITY

(PROGRESS REPORT: 1 JUNE 2000 THROUGH 31 MAY 2001)

by

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Introduction

A major focus of the captive broodstock research group is to improve the quality of adult salmon grown in captive broodstocks through manipulation of environmental parameters (e.g., diet, feeding regimens, and exercise). This research requires a substantial culture facility containing the numbers of tanks for valid experimental replication, and the tanks and water supply must be adequate to support culture to maturity. The facility must also provide for the maximum of experimental control of potentially confounding factors. Currently, no such facility is available for such research.

The NMFS Manchester Research Station currently supplies filtered, sterilized sea-water to populations of ESA-listed chinook and sockeye salmon. Effluent from these tanks is available for use in culturing salmon captive broodstocks for research on adult quality. The fish culture system at Manchester will be expanded to accommodate 12 large, identical rearing vessels dedicated to research. This approach takes advantage of the existing infrastructure and realizes cost savings over development of a completely new facility.

A major goal of the Captive Broodstock Research Project (current and future) is determining the effects of experimental rearing strategies (such as dietary manipulations, growth regimens, exercise, therapeutic disease treatments) on survival, and the quality of adults for release and artificial spawning. Such research has been identified as high priority by the various technical committees overseeing current captive broodstock programs for ESA-listed salmon. This complex and integrated research requires populations of experimental fish, together with the investment in infrastructure, to carry

out valid comparative long-term experiments. Manchester has the quarantine ability to take and return large volumes of seawater to Puget Sound, because of a unique ozone depuration system which operates full-time.

A further advantage of expanding the research resources at Manchester is that it will be integrated with one of the major captive broodstock rearing facilities for ESA-listed stocks. This will increase the capacity for information exchange between ongoing research and practical stock restoration.

Approach

The basic design of the proposed facility is as follows. Effluent seawater (approximately 2,500 L/min) from the ESA-listed captive broodstocks at Manchester will be collected in a concrete vault. Approximately 2,000 L/min of the water will be pumped, sand-filtered, UV-sterilized, and delivered to an array of 12 large rearing tanks situated on a concrete pad. The water will exit the tanks, enter open drains in the concrete pad, and enter the existing ozone depuration system before being discharged into Puget Sound.

Work Completed

In 1999, a concrete sump, submersible pumps, and some plumbing were purchased and installed to collect and distribute water to the proposed facility. Other necessary equipment, such as sand filters and UV sterilizers, was purchased but not installed.

Last year (2000) saw the completion of the following tasks:

- Installation of two sand filters, each capable of filtering > 2,000 L/min
- Installation of a UV-sterilizer, capable of disinfecting 2,500 L/min
- Preparation and construction of a concrete pad with open drains
- Installation of the necessary plumbing to supply water to the tank pad and return effluent to the ozone depuration system, and
- Installation of 12 rearing tanks (4.4 m diameter)

Work to be Completed

Some small electrical and plumbing tasks remain to be completed. The two pumps will be wired for installation. The main pump will be controlled by a variable frequency electrical controller, and the second (back-up) pump wired only for simple on/off operation. The last of the plumbing of the rearing tanks will be completed, and covers will be constructed to reduce light and hence algal growth.

The complete system should be functional by 30 July 2001, at which time chinook captive broodstocks will be transferred to the tanks in anticipation of research experiments to be conducted in 2003 and 2004.

TASK 9. THE EFFECT OF GROWTH RATE ON DISEASE RESISTANCE

(PROGRESS REPORT: 1 JUNE THROUGH 31 MAY 2001)

by

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Introduction

There is evidence that many of the specific and nonspecific host defenses of salmonids and other fishes are affected by some fish culture practices. One objective of research conducted under Project 93-56 during 1999 was to measure the effect of growth rate on the immune health of chinook salmon. Results suggested that certain functions of a chinook salmon's phagocytic cells become impaired as the ration (and growth) is increased. These cells are part of the fish's first line of defense against many pathogenic microorganisms, and any improper modulation of their activity attributable to the rate of feeding must be carefully assessed with regard to disease resistance. Whereas these results may be important to the fish culturist, the understanding of the relation between growth rate and disease resistance remains incomplete.

The objective of the Task for 2000 was to verify the significance of the 1999 results by comparing the same indices of nonspecific and specific immunity among groups of chinook salmon fed ration amounts calculated to reduce growth rates even further than the lowest rate used in that study. A high-growth-rate control group is now included in the new study to ensure evaluation of the widest range of growth rates possible under the experimental conditions. At regular intervals, fish from each growth-rate group are to be sampled to measure immune parameters. Subgroups are also be immunized with a purified protein antigen to determine if growth rate effects their relative abilities to produce an antibody response.

Research Plan

The study is examining more closely the effects of differing growth rates by producing groups of juvenile chinook salmon of sizes similar to their wild counterparts.

It is using fish from three of the (6) groups described in (Task 6) to study the effect of growth rate on early male maturation. The use of the same fish to investigate the effect of differing growth rates on disease resistance and early sexual maturation of males simultaneously will strengthen the analyses of both assessments. Further evaluation will determine if the growth rate of chinook salmon affects selected indices of their nonspecific immunity, and if changes in the growth rate also affects their ability to produce an antibody response to a foreign protein.

Experiment A. Feed groups of chinook salmon at different rates to regulate growth.

Work Completed and to be Completed

The experimental fish are brood year 1999 spring chinook salmon from the Willamette River stock, OR, and are being reared at the Northwest Fisheries Science Center. Beginning in February 2000, for each feed-rate group, fish in replicate tanks were fed a commercial high-fat diet (BioOregon, Warrenton, OR) at differing rates such that the fish in a given group reached a predetermined mean weight during December 2000. Only fish from feed-rate groups A, E, and F are being used (see Work Statement) and fed at the appropriate experimental rates until February to September 2001.

Experiment B. Compare the relative abilities of the different feed-rate groups to respond to a protein antigen.

Work Completed and to be Completed

This experiment is ongoing and on schedule. Subgroups of fish from feed-rate groups A, E, and F were injected intraperitoneally with 100 µg of fibrinogen in Freund's complete adjuvant to measure their relative abilities to produce a humoral response. Ten fish from each inject group were bled 6, 7, 8, 9, and 10 weeks after injection. Serum samples were stored at -70°C for analysis by an enzyme-linked immuno-sorbent assay for fish immunoglobulin.

Experiment C. Compare indices of nonspecific immunity among the different feed-rate groups.

Work to be Completed

For each measurement of the nonspecific immune functions, subgroups of 30 fish will be randomly chosen from each feed-rate group. A total of 15 fish will be tested from each replicate tank of the appropriate feed-rate groups. Five fish will be removed from each tank by dip-net every other day for 5 days. Fish will be anesthetized with tricaine methanesulfonate (MS-222). The weight and fork length will then be determined to calculate the condition factor. Condition factor values are corrected for the use of metric measurements by the formula $C = 36.14K$, where C and K are condition factors based on English and metric units, respectively.

A blood sample will be taken by caudal vein puncture for the hematocrit, leucocrit, and plasma protein determinations. Duplicate smears of whole blood will be made for individual fish. The remainder of each sample will be allowed to clot overnight at 4°C and then centrifuged at 5,000 × g for 20 min. The serum will be stored at -80°C.

On each sample date, a five fish-pool of anterior kidney tissue will be prepared from each subgroup. The anterior kidney will be aseptically removed and a small portion from the center of the sample used to make several kidney imprints on each of two glass microscope slides. The tissue imprints will be air-dried, then fixed in absolute methanol for 5 minutes. One of the slides will be stained for differential cell counts, and the other slide stored at 4°C for the myeloperoxidase assay. Leucocytes will be purified from the remaining tissue in each pool by gradient centrifugation for use in the phagocytosis and NBT assays.

TASK 10. USE OF ANTIBIOTICS AND NEW VACCINES TO REDUCE MORTALITY FROM BACTERIAL KIDNEY DISEASE IN CHINOOK SALMON

(PROGRESS REPORT: 1 JUNE 2000 THROUGH 31 MAY 2001)

by

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Introduction

Bacterial Kidney Disease (BKD) is the major infectious disease affecting the successful culture of salmonids in the Pacific Northwest. In 1993 and 1994 this disease was responsible for catastrophic losses of endangered Redfish Lake sockeye salmon held as captive broodstock. BKD-caused epizootics continue to impact captive rearing programs of both sockeye and chinook salmon (Schiewe et al. 1997).

The long-term prophylactic administration of erythromycin is commonly used to reduce losses due to BKD. Results of preliminary studies with Lake Wenatchee sockeye salmon suggest that erythromycin may have a negative effect on gamete viability. Recent experiences with Catherine Creek, Lostine River, and Lemhi River spring chinook salmon have shown currently mandated treatment regimens may elicit fatal toxicity reactions. Until an effective vaccine becomes available, erythromycin and/or azithromycin will continue to be used. The approach is to design treatment regimens

which avoid these toxic reactions and reductions in reproductive success. It is first necessary to determine the causes of the reactions and conditions under which they appear. A long-term study on potential toxic reactions after prophylactic use of erthryomycin and azithromycin is underway, together with the verification that limited prophylactic use of the more experimental azithromycin in captive broodstock rearing programs does not result in its release in effluent. In addition, vaccine trials will be initiated to determine the efficacy of a new commercial BKD vaccine as well as experimental vaccine preparations to induce a protective immune response.

There is no vaccine available to protect salmon from infections with *Renibacterium salmoninarum*, the causative bacterium of BKD. Erythromycin has been the primary antibiotic used by fish culturists in an attempt to prevent and control *R. salmoninarum* (Elliott et al. 1989), administered orally through feed or by injection of maturing adults. However, while use of erythromycin usually results in short-term health improvement of infected fish, it fails to eliminate the infection and symptoms of disease completely, which often return after ending treatment. In addition to erythromycin another macrolide antibiotic, azithromycin, has been tested for the treatment of BKD in captive broodstock salmon. In experiments carried out between 1996-1999 under the BPA Captive Broodstock Research Program, azithromycin was shown to have a strong bactericidal activity against *R. salmoninarum* both *in vitro* and *in vivo*, and is effective in reducing clinical symptoms of BKD, improving long-term survival through spawning. The effectiveness of the drug probably lies in its ability to concentrate in polymorphonuclear leukocytes, macrophages, and fibrocytes (Peters et al. 1992), all cell types that *R. salmoninarum* is known to invade and be sequestered from the salmonid immune system (Bandin et al. 1993; Gutenberger et al. 1997).

Long-term prophylactic use of antibiotics should be avoided where possible. In order to reduce antibiotic treatment of BKD, effective vaccines or other non-antibiotic-based therapeutics will have to be developed and used. A number of experimental vaccines have been formulated and tested, but none has proven effective enough for general use. These have included whole-killed cells, or bacterins, heat-treated whole fixed cells (to reduce the amount of p57 protein on the surface, a putative major virulence factor), and purified p57.

Aqua Health Limited recently licensed a vaccine (Renogen) for use with Atlantic salmon in Canada. This vaccine is a heterologous vaccine, which consists of a preparation of the bacterium *Arthrobacter sp. nov*. It appears to induce a cross-reacting immune response which offers protection against *R. salmoninarum* infection. The bacterium is administered as a live culture, delivered in an intraperitoneal injection. The vaccine has undergone safety testing in chinook salmon presmolts at the Idaho Department of Fish and Game Eagle Fish Health Laboratory in early 2000. Under this project, the vaccine will be administered to chinook salmon presmolts. After smolting, the vaccinated fish will be experimentally challenged with a virulent strain of *R. salmoninarum* to determine the efficacy of the vaccine to protect chinook salmon from BKD. Besides Renogen, preliminary vaccine trials using attenuated strains of *R.*

salmoninarum currently under study by the Northwest Fisheries Science Center will also be initiated under this proposal.

Experiment A. The Efficacy of the Vaccines

Work Completed

The main objective of this study is to determine the safety of vaccination of chinook salmon with the commercial vaccine Renogen, and subsequent protection against bacterial kidney disease. A sub-objective is to initiate vaccine trials with an attenuated *R. salmoninarum* strain. The approach was to vaccinate test fish and then infect them with virulent *R. salmoninarum* by intraperitoneal injection and measure the efficacy of the vaccine.

Approximately 2000 Minter Creek fall chinook salmon (~ 5 g) were selected for the study, and were pre-vaccinated against *Vibrio* after smolting (Alpharma Alpha Dip 2100 *Vibiro anguillarum-ordalii* bacterin, delivered by intraperitoneal (IP) injection). At the same time, fish designated for vaccination with Renogen, were also injected with the Renogen vaccine following the manufacturer's directions (Aqua Health Ltd., Canada). Control fish were mock-vaccinated with PBS. Two weeks post-vaccination, the fish were transferred and acclimated in pathogen-free seawater at the Manchester Research Station for four weeks prior to *R. salmoninarum* challenge. Fish were fed a standard unmedicated diet. During adaptation, randomly selected fish were screened for the presence of BKD by ELISA and FAT of kidney tissues, and by RT-PCR of blood samples (Rhodes et. al. 1998).

Two Renogen efficacy trials were carried out. In the first, fish consisted of two groups, Renogen-vaccinated and mock-vaccinated. Three hundred Renogen-vaccinated and 300 mock-vaccinated fish were injected IP with 1 x 10^6 *R. salmoninarum* bacteria (1 x 10^5 bacteria/g fish), and placed in three circular 1.6-m tanks, 100 fish/tank. Likewise, 200 Renogen and mock-vaccinated fish each were mock-challenged with PBS as controls, and divided into two tanks for each group. The health of the fish was closely monitored over time (~3 months). Dead and moribund fish were removed immediately, necropsied, and examined for typical BKD lesions, and FAT and ELISA tests were performed on kidney tissue.

The second Renogen trial followed essentially the same protocol as the first with the exception that the challenge bacterial dose was reduced to ~1 x 10^4 *R. salmoninarum*/g fish, with each treatment performed in duplicate.

In addition to the Renogen vaccine trials, two additional trials were carried out using fixed (killed bacterin-type) and live attenuated *R. salmoninarum* strains as vaccines. In the first of these trials, two groups of 50 fish each were vaccinated with fixed or live *R. salmoninarum* MT-239 (an attenuated or less virulent strain under study in our laboratory); an additional two groups of 50 fish were not vaccinated as a control. Approximately 30 days post-vaccination, one group of each were challenged IP with 1 x

10^4 wild-type *R. salmoninarum* (~1.2 x 10^3 bacteria/g-fish) while the other group were mock-challenged with PBS. In the second trial, two groups of 45 fish each were vaccinated with fixed MT239, live MT239, and fixed wild type *R. salmoninarum* (strain 33209); again, ~ 1 month after vaccination, the fish in one group of each pair were challenged IP with 1.3 x 10^6 wild-type *R. salmoninarum* (~1.4 x 10^4 bacteria/g fish). As with the Renogen trials, the health of the fish was closely monitored over time (~3 months). Dead and moribund fish were removed immediately, necropsied and examined for typical BKD lesions, and FAT and ELISA tests were performed on kidney tissue.

Work to be Completed

Most samples from all the vaccine trials have been analyzed for FAT or ELISA values, with the remaining samples to be completed by July, 2001. Stringent statistical analysis on the data still needs to be performed before broader conclusions can be drawn. A final report incorporating data from all of the vaccine trials completed under this work statement will be prepared for the June 2001-May 2002 reporting period.

Experiment B. Determine Azithromycin Depuration or Inactivation Rates in Effluent from Salmon During Feed-based Antibiotic Treatment

Work to be Completed

This experiment will be carried out with fish that have survived the various vaccine-trials under the 2000-2001 work plan at the Manchester Research Station pathology laboratory, and prior to setting up the laboratory for vaccine trials with new fish under the 2001 - 2002 work plan.

Experiment C. Evaluation of Toxic Effects of Long-term Prophylactic Use of Azithromycin and Erythromycin

Work Completed

The three main objectives of the experiment are: (i) Determine how captive fall chinook salmon broodstock gonad development, gamete viability, and survival of the progeny though the swim-up stage are affected by long-term prophylactic administration of erythromycin or azithromycin; (ii) measure (if the experimental fish are found to be naturally infected with *R. salmoninarum*,) the effects of two erythromycin treatment regimens (two or four treatments annually) on BKD incidence from the first feeding fry through mature adult life history stages; and (iii) determine whether erythromycin toxicity responses are related to treatment frequency. This aspect of the study will include a measurement of residual tissue concentrations and will document underlying tissue damage associated with the syndrome. While the study focuses on erythromycin, as it is the current antibiotic of choice and the one apparently causing the severe toxic side effects, the long-term effects of azithromycin treatment will also be assessed concurrently.

Phase 1: First feeding to smolt

During January 1999, approximately 3,150 Big Beef Creek fall chinook salmon were transferred to the Big Beef Creek Hatchery. Prior to initiation of exogenous feeding, 400 fish were randomly stocked into each of 14 isolation tanks (two tanks per treatment). Using this experimental design, the following feedings were carried out:

- No treatment.
- Erythromycin administered orally at a rate of 100 mg/kg/d for 28 d, or azithromycin administered orally at a rate of 30 mg/kg/d for 14 d, the first treatment administered at initiation of exogenous feeding, the second during sexual differentiation (2 g average wt), and the third just prior to smoltification (ca. 7 g average wt).
- Erythromycin administered orally at a rate of 100 mg/kg/d for 28 d, or azithromycin administered orally at a rate of 30 mg/kg/d for 14 d, the first treatment administered during sexual differentiation (2 g average weight) and the second just prior to smoltification (ca. 7 g average weight).
- Erythromycin administered orally at a rate of 100 mg/kg/d for 28 d, or azithromycin administered orally at a rate of 30 mg/kg/d for 14 d, with the only treatment administered just prior to smoltification (ca. 7 g average weight).

At the beginning of the trial and following each treatment, fish from each experimental tank were collected for measurement of tissue antibiotic concentrations and for histological evaluation of brain, heart, kidney, liver, spleen, stomach, pyloric cecae, and intestine for abnormalities associated with erythromycin or azithromycin toxicities. The collected specimens are currently being analyzed following methods described in the work plan.

Phase 2: Smolt to maturity

Phase 2 of the study was initiated in June 1999. One hundred twenty fish from each tank were PIT tagged and divided randomly into three groups of 40 fish each, which were combined as follows:

Tank 1: Forty fish from Treatments 1-7, replicate A
Tank 2: Forty fish from Treatments 1-7, replicate A
Tank 3: Forty fish from Treatments 1-7, replicate A
Tank 4: Forty fish from Treatments 1-7, replicate B
Tank 5: Forty fish from Treatments 1-7, replicate B
Tank 6: Forty fish from Treatments 1-7, replicate B

Two tanks of fish were then assigned to each of the experimental treatments. Using this experimental setup, the following feedings are being carried out:

- No treatment.
- Erythromycin administered orally at a rate of 100 mg/kg/d for 2 d, twice per year until mature (November-December 2001).
- Erythromycin administered orally at a rate of 100 mg/kg/d for 28 d, four times per year until mature (November-December 2001).

To date, the erythromycin treated groups have received either one (of two), or three (of four) treatments scheduled for the 2000-2001 reporting period.

During August 2000, fish from each experimental tank were collected for measurement of tissue antibiotic concentrations, and for histological evaluation of brain, heart, kidney, liver, spleen, stomach, pyloric cecae, and intestine for abnormalities associated with erythromycin or azithromycin toxicities. The collected specimens have been preserved and will be analyzed at the end of the study following methods described in the work plan.

Work to be Completed

Phase 1: First feeding to smolt stage

More than 90% of the samples collected during this phase have been analyzed to determine the concentration erythromycin or azithromycin in the tissues. Preliminary histological examination of fish subjected to the experimental treatments has been completed. Additional samples will be evaluated to confirm the preliminary results. All data will be reported along with Phase 2 at the end of the FY01 performance period

Phase 2: Smolt to maturity

Samples will be collected in September 2001 to evaluate the effects of the various treatment regimens (i.e., pre- and post-smolt treatments) on BKD prevalence, drug clearance rates, and organ histology. Maturing females will be spawned towards the end of 2001 to evaluate the effects of the various treatment regimens on reproductive success. Laboratory work will be completed by June 2001. The final report will be prepared at the end of the FY01 performance period.

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TASK 11. INBREEDING

(PROGRESS REPORT: 1 JUNE 2000 THROUGH 31 MAY 2001)

by

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Introduction

Many wild salmon populations exist in low abundances. It is not yet known to what extent inbreeding has led to and continues to impede productivity of these populations, which aspects of the life cycle are affected most, and whether inbreeding can limit the effectiveness of recovery efforts involving hatchery supplementation or captive broodstocks.

Inbreeding depression, a reduction in fitness caused by the mating of close relatives, has for decades been among the most prominent genetic concerns of captive breeding programs involving threatened or endangered species. This concern stems from adverse effects of inbreeding on survival and reproductive capacity that have been well documented in many species of captively-bred animals (Ralls 1983), and experimental work has shown a strong link between the degree of inbreeding and fitness loss (Ralls et al. 1988). A recent study (Saccheri 1998) clearly demonstrated that reduced genetic variation associated with inbreeding can contribute directly to extinction of wild populations. Furthermore, evidence is mounting that a past history of inbreeding (e.g., due to historically small population size) does not necessarily buffer a population from subsequent inbreeding depression (Ballou 1997). The consequences of inbreeding in most salmonids are poorly understood. The relevant work has been limited to non-anadromous fish, especially brook and rainbow trout. Nevertheless, studies on these species have found adverse effects of close inbreeding on survival and growth (Hard and Hershberger 1995), and a current review of these studies provides evidence that these effects may occur in other anadromous salmonids as well (Wang et al., in preparation).

Even if inbreeding depression leads to higher risk of extinction, it is difficult to evaluate this risk relative to other risks, such as catastrophic loss or domestication of

animals in captivity, and population fragmentation or local extinction in the wild. This is particularly true in light of recent evidence that inbreeding depression may reduce fitness sharply at intermediate levels of inbreeding (Frankham 1995) and its extent is likely to vary in different environments (Pray 1994).

Research on the consequences of inbreeding in anadromous salmonids would be most useful in characterizing the relationship between inbreeding and inbreeding depression, and the environmental sensitivity of inbreeding depression. For captive broodstock programs, this information would help to evaluate the risk of inbreeding depression against other risks (such as the risk of domestication); this in turn would help to formulate guidelines for determining:

(i) under what population scenarios a captive broodstock or captive rearing program should (and should not) be initiated based on current inbreeding levels,

(ii) what captive population sizes should be maintained, and for how many generations, and

(iii) what characteristics of the captive environment are most important to simultaneously reduce risk of inbreeding depression and domestication.

Approach

This is an ongoing research project, and three basic hypotheses are currently being tested:

H_{01}: Inbreeding depression does not reduce viability or alter life history characteristics of chinook salmon.

- H_{a11}: Inbreeding depression reduces viability during early life history but does not affect development rate, age structure, or reproductive capacity.
- H_{a12}: Inbreeding depression has effects throughout the life cycle.

H_{02}: The degree of inbreeding has no predictable effect on inbreeding depression in chinook salmon.

- H_{a21}: The relationship between inbreeding and inbreeding depression is linear.
- H_{a22}: The relationship between inbreeding and inbreeding depression is nonlinear.

H_{03}: Inbreeding depression in chinook salmon does not vary between captive (i.e., protective culture throughout life cycle) and hatchery (i.e., protective culture from embryo to smolt) environments.

- H_{a31}: Inbreeding depression is greater in a hatchery than in a captive environment.
- H_{a32}: Inbreeding depression is greater in a captive than in a hatchery environment.

In 2000-2001, work continued to address these stated hypotheses by conducting genetic analyses of biological data from first-generation adults returning to their site of release and from experimentally inbred and control captively-reared progeny cultured in marine netpens. Work is ongoing in Puget Sound, Washington at the NMFS Manchester Research Station (MRS), the site of captive-rearing in marine net-pens, and the

University of Washington's School of Fishery and Aquatic Sciences Hatchery (UWH), the site of releases of inbred progeny. One complete generation of experimental inbreeding will be accomplished with the maturation of adult inbred progeny in October 2003. This report briefly summarizes current progress for this study.

Work Completed

To date this study has:

- Established, using a conventional quantitative genetic breeding design, an experimental hatchery population (Grovers Creek Hatchery, Puget Sound, Washington) of 1994-brood fall chinook salmon structured of 96 full-sib families nested within 30 half-sib families;
- Determined the genetic and environmental components of variation in body morphology of these fish at the parr-smolt stage of development (Hard et al. 1999);
- In 1995 released to sea 257,093 of these fish from Grovers Creek Hatchery, each identified with full-sib family-specific coded-wire tags;
- Cultured several hundred 2- , 3-, and 4-year-old fish marked individually with Passive Integrated Transponder (PIT) tags from the same cohort to maturity in marine net-pens at MRS;
- Spawned several hundred adults returning from the 1995 releases or maturing in the marine net-pens to establish 3 experimentally inbred lines from progeny of the 1994-brood parents at UWH;
- Established 300 1997-brood PIT-tagged smolts from a total of 27 families of 3-year-old captive adults in the inbred lines in seawater net-pens at MRS;
- Established at MRS 1,500 1998-brood PIT-tagged smolts from a total of 75 families of 4-year-old hatchery adults in the experimentally inbred lines. The experimentally inbred lines correspond to a minimal increment in inbreeding (randomly mated control), a moderate increment in inbreeding (half-sib parents, corresponding to an approximate increase in inbreeding of 12.5%), and a substantial increment in inbreeding (full-sib parents, corresponding to an approximate increase in inbreeding of 25%); and
- Collected biological and coded-wire tag information from adults returning to UWH in 1999 and 2000. The tags are currently being decoded. No matings in either year from these fish were constructed because too few experimental females could be identified for a full mating design.

Preliminary data on the effects of one generation of inbreeding, measured on stage-specific survival and growth during early life history, were summarized in a previous report covering three years of work between 1996 and 1999. These data are undergoing final analyses, which will be summarized in the annual report due June 2002.

Maturation of 1994-brood parents

Data collection for 1994-brood adults (the parents of the fish inbred for a single generation) is now complete. These fish are the last of the first-generation progeny of the

broodstock collected in 1994 to establish this study. They represent the completion of the half-sib/full-sib family breeding design instigated with the 1994 return to Grovers Creek Hatchery. As indicated in previous reports, this breeding design is routinely used in animal and plant breeding to estimate genetic parameters that describe a population's ability to respond to selection and other forces (Falconer 1989; Lynch and Walsh 1998). The design permits estimation of genetic and environmental components of variance for a variety of phenotypic traits, as well as providing a convenient means of establishing different levels of inbreeding in experimental groups within the population. This design is described in detail by Hard and Hershberger (1998) and Hard et al. (1999). Hard and Hershberger (1998) also summarized much of the data collected from the source parents collected at Grovers Creek Hatchery in 1994.

Maturing 1994-brood adults (the F_1 progeny of these parents) were sampled in September and October each year from 1995 to 1999 as they returned to Grovers Creek Hatchery. From these returns marked fish were sampled using body size and the absence of an adipose fin to determine which fish were associated with the inbreeding experiment. Data collected included age, sex, fork length, and round weight, as well as family-assignment information from their coded-wire tags, decoded *in situ* before spawning. For females, volumetric measurements of the egg mass were made and individual samples of eggs for egg-size measurements were collected to estimate fecundity. In addition, tissue samples were collected for later genetic analysis and three digital photographs of each adult for analysis of morphometric variation. Milt from most experimental males was cryogenically preserved. Experimental fish were mated and families established in incubation units at UWH and these F_2 progeny were reared to the smolt stage, when they were marked with group-specific coded-wire tags, and either released from UWH in 1998 and 1999, or a number from each family were marked with individual PIT tags for culture to adulthood in marine net-pens at MRS.

These data will be summarized in next year's annual report. Meanwhile, a genetic analysis of several of the adult characters has been drafted, measured on 629 F_1 adults returning to Grovers Creek Hatchery from 1995-1999, in an attempt to determine how these characters would respond to various forms of selection. A preliminary analysis of these data using Restricted Maximum Likelihood (REML) methods (Hard et al. 1999) yielded the following estimates of heritability for these characters (see Table 1):

Table 1. Estimates of heritability of adult characters (with standard errors for REML estimates). Approximate standard errors in parentheses; n = 629 for all traits. Transformations of these characters to reduce departures from normality were the cube of age, the square root of weight, and natural logarithm the date components of both spawn date and growth rate.

Character	$h^2 \pm SE$
Fork length (mm)	0.336 ± 0.208
Adult age (yr)	0.349 ± 0.212
Adult weight (g)	0.010 ± 0.091
Spawn date (Julian d)	0.232 ± 0.172
Growth rate (mm $(d)^{-1}$)	0.307 ± 0.198

These data indicate that detectable genetic variation exists for several important life history characteristics in this population. It is planned to use these estimates, obtained from the covariance among relatives, to parameterize age-structured models of multivariate response to selection. To this end, the genetic and phenotypic covariance matrices for these traits is being computed, and draft manuscripts are being prepared which will examine how the life history of populations with these genetic characteristics are likely to respond to different forms of harvest selection over short-term evolutionary time.

Analysis of experimentally inbred progeny

Between June 1998 and January 2001, 1997- and 1998-brood F_2 chinook salmon marked with PIT tags were raised in marine net-pens at MRS (corresponding fish from these groups had been released from UWH in 1998 and 1999). The fish were fed 2 - 4 times daily, at an average feeding rate which varied from 1.5 - 3.0% mean body weight per day. Fish were sampled every 3 - 4 months during this period to estimate survival, length, and weight. By June 1999, two broods of experimentally inbred fish were under culture at MRS. Unfortunately, marine mammals (mainly seals and sea lions) had killed the few (<100) 1994-brood fish remaining at Manchester during winter 1998 - 1999, and river otters at the site eliminated the 1997-brood population in February 2000 and the 1998-brood population in January 2001.

For each PIT-tagged brood, survival was computed directly from the number of live fish at each sampling event, as all fish were counted and measured during each sampling event and sampling was with replacement. Fish length (fork length to the nearest mm) and weight (wet weight to the nearest g) was measured for each fish in the census. For both populations, lengths and weights of all fish in a sample were measured but, where necessary, some weights from linear regression of weight on length were estimated. All PIT-tagged fish were weighed and measured during each census.

These data are currently being analyzed for results to be presented next year.

Publications

In addition to these analyses, three papers supported by this project were submitted for publication in 2001. These are:

Wang, S., J.J. Hard, and F.M. Utter. Salmonid inbreeding: a review. Reviews in Fish Biology and Fisheries. This paper summarizes the current state of knowledge of inbreeding and its consequences in salmonid fishes.

Wang, S., J.J. Hard, and F.M. Utter. Genetic variation and fitness in salmonids. Conservation Genetics. This paper summarizes the state of knowledge of the relationship between genetic variability (e.g., heterozygosity at putatively neutral genetic markers, frequency of rare alleles) and measures of fitness and performance in salmonid fishes.

Wang, S., J.J. Hard, and F.M. Utter. Effects of inbreeding on genetic variation, early survival, and developmental stability of chinook salmon (tentative title). Aquaculture. This paper summarizes experimental work on chinook salmon, testing observed rates of inbreeding against those expected from theory.

Work to be Completed

Work in 2001-2002 will entail the following elements:

- Collection of experimentally inbred and control F_2 1997- and 1998-brood Grovers Creek stock chinook salmon returning to UWH in September and October 2001 (these fish will be continually collected as they return to UWH each year until 2003);
- Mating of these fish to perpetuate the experimental lines by creating second-generation inbred (F_3) fish; and
- Culture of the resulting F_3 progeny to the smolt stage in freshwater, followed by release of most fish from UWH, with representatives of each family to be held captive in seawater at the Center's field research stations at Manchester or Mukilteo.

Depending upon the number of adults which survive to return to UWH, up to 35 full-sib families will be established in each of the experimental lines (randomly mated, half-sib mated, and full-sib mated). Experimental groups will be created at the time of spawning after decoding group-specific coded-wire tags at UWH. Experimental matings will be made at the end of each spawning day, and culture progeny to the smolt stage at the hatchery. Parents of known lineage from within each line were mated together at random on each spawning date to establish the second-generation experimental groups.

The following information will be collected and analyzed for three- and four-year old 1997- and 1998-brood adults maturing in autumn 2001 and returning to UWH: survival, body length and weight, and for any maturing females, egg size and weight or volume of the egg mass (as a proxy for fecundity). For their progeny, data will be collected through the smolt release in June 2002 on stage-specific survival, growth and development rate, and meristic and morphometric variation. Smolts will be marked with group-specific coded-wire tags, with most fish released from the hatchery to sea and up to 5,000 (with up to 50 representatives from each full-sib family) PIT-tagged for grow-

out to adulthood in captivity. Collectively these data will provide a preliminary assessment of second-generation inbreeding effects on early life-history traits and, through examination of the control population, an assessment of relative environmental influences in consecutive generations on these traits.

For next year's annual report, analysis of inbreeding depression after one generation of inbreeding will be based upon the data collected from 1997- and 1998-brood fish up to 3 years old; this analysis should be complete by May 2002. It is expected that evaluations to determine if the three experimental lines differ in stage-specific survival, growth, development rate, and meristic and morphometric variation will be complete by this time. However, the evaluations await confirmation of the experimental pedigree with DNA micro-satellite analyses being completed by Shizhen Wang of the UW, and final collection of meristic and morphometric data from 1998-brood progeny sampled from each full-sib family in each experimental group by Kathleen Neely of the NWFSC.

General linear models and analyses of variance in survival and early growth within and among lines will be used to test the hypotheses stated above and evaluate the effects of inbreeding on these characters, following methods developed by Lynch (1988) and Lynch and Walsh (1998) and described in Hard and Hershberger (1998). If necessary, these tests will be followed with retrospective power analyses to identify the magnitudes of observed differences necessary to detect significant inbreeding depression (Cohen 1988). It is planned to compare survival and growth among the three experimental groups, (i) progeny of parents mated at random but excluding matings between siblings (control), (ii) progeny of full-sibling parents (corresponding to an average increase in the inbreeding coefficient, F, of 0.25 in one generation), and (iii) progeny of half-sibling parents (corresponding to an average increase in F of 0.125 in one generation). For each trait the coefficient of inbreeding depression (Lande and Schemske 1985) will be computed to provide direct comparison of inbred and control groups. Where it is possible to do so from the survival data, the number of lethal equivalents per gamete together with the cost in survival associated with one generation of full-sib mating will be estimated using the methods described by Morton et al. (1956), Ralls et al. (1988), and Kalinowski and Hedrick (1998).

If sufficient adults are available, it is planned to establish an additional set of experimentally inbred lines with chinook salmon from the UWH broodstock. This set of lines would serve two purposes. First, it would provide an independent replicate for the entire inbreeding study, using a distinct but closely related population for the experiment. Second, it would provide a safeguard against complete loss of Grovers Creek fish due to catastrophic failure at one of the culture facilities, or predation during the marine culture phase. This set of lines would also provide an opportunity to examine the effects of inbreeding and outbreeding operating simultaneously in chinook salmon. For the UWH replicate experiment, a hierarchical breeding design will replicate that described by Hard et al. (1999).

A summary of the comprehensive genetic and phenotypic analyses of the biological data collected from the parents will be summarized in the next report. It is planned to submit a final report on this project when one full generation of experimental inbreeding is implemented with the maturation of adult inbred progeny through age 5, in June 2004.

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APPENDIX A.

TASK 10. TOXICITY OF PROPHYLACTIC TREATMENTS FOR BACTERIAL KIDNEY DISEASE (BKD)

(FY 1999 FINAL REPORT: 1 JUNE 1999 THROUGH 31 MAY 2000)

by

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Introduction

Bacterial kidney disease (BKD) is the major infectious disease preventing successful culture of salmonids in the Pacific Northwest. Between 1993-94 this disease was responsible for catastrophic losses of endangered Redfish Lake sockeye salmon held as captive broodstock. Long-term prophylactic administration of erythromycin has become common in recent years to reduce losses due to BKD. Results of preliminary studies with Lake Wenatchee sockeye salmon suggest that erythromycin may have a negative effect on gamete viability. Also, recent experiences at Manchester Research Station with Catherine Creek, Lostine River, and Lemhi River spring chinook salmon have shown currently mandated treatment regimens may elicit fatal toxicity reactions.

Until alternative therapeutics, such as azithromycin, are identified and proven efficacious for treating BKD in captive broodstock, or until an effective vaccine becomes available, use of erythromycin will continue.

BKD-caused epizootics continue to significantly impact captive rearing programs of both sockeye and chinook salmon (Schiewe et al. 1997). There is no vaccine available to protect salmon from infections with *Renibacterium salmoninarum*, the causative bacterium of BKD. Erythromycin has been the primary antibiotic used by fish culturists in an attempt to prevent and control *R. salmoninarum* (Elliott et al. 1989). It is administered orally through feed or by injection of maturing adults. However, while use of erythromycin usually results in short-term health improvement of infected fish, it fails to eliminate the infection completely.

The symptoms of BKD often return after treatment ends. Although prophylactic treatment of spawning females does seem to reduce some of the vertical transmission of *R. salmoninarum* to eggs (Evelyn et al. 1986a, Bullock and Stuckey 1986), in general this practice has not led to satisfactory results. There are no proven vaccines available to prevent BKD in Pacific salmonids, and there is a clear need for alternative chemotherapeutics. Azithromycin is one such candidate, a new macrolide antibiotic with the same mode of action as erythromycin, and which actively kills *R. salmoninarum* bacterium *in vitro*. This new antibiotic concentrates in polymorphonuclear leukocytes, macrophages and fibrocytes (Peters et al. 1992), cell types *R. salmoninarum* has been shown to invade, which in turn protect the organism from the host humoral immune system (Bandin et al. 1993; Gutenberger et al. 1997). Azithromycin has strong bactericidal activity against *R. salmoninarum in vitro* and has shown efficacy *in vivo* in prior experimental BKD infections of salmonids.

Most antibiotics, including erythromycin, do not penetrate tissues well. After oral or parenteral administration they are bound to serum protein and remain in extracellular spaces. By contrast, azithromycin is rapidly absorbed in tissues and distributed at higher concentrations in cells instead of in plasma or serum, with a longer active half-life (Peters et al. 1992).

Azithromycin has been shown effective in reducing the intracellular viability of nearly all invasive bacterial species tested. In general it is more effective than erythromycin against Gram-negative bacteria, but is only marginally less active against Gram-positive organisms (Peters et al. 1992). Even then, the latter is of doubtful clinical significance because of the higher and longer-acting issue concentrations that can be achieved with azithromycin. This view is supported by reports that show that azithromycin is somewhat more effective than erythromycin in reducing intracellular (invasive) enteric pathogens that were phagocytized by neutrophils (Rakita et al. 1994). The authors suggested that the concentration of azithromycin in neutrophils may be particularly useful in treating infections caused by invasive pathogens which multiply intracellularly in host cells.

Numerous studies report greatly increased intracellular uptake and superior antibacterial activity of azithromycin over erythromycin *in vitro* and *in vivo* with animals other than fish (Peters et al. 1992). Other work compared the intracellular activity of azithromycin and erythromycin against an intracellular protozoan parasite, *Toxoplasma gondii*, and reported superior performance of azithromycin (Lode et al. 1996). Azithromycin accumulated readily and remained inside macrophages infected with the protozoan, interfering with growth of the parasite (Schwab et al. 1994). While there are no published studies demonstrating the efficacy of azithromycin in fish, it's broad spectrum activity and ability to cross into tissues make it an important antimicrobial to test for its ability to control *R. salmoninarum*.

The efficacy of the two macrolide antibiotics, erythromycin and azithromycin in treating BKD in chinook salmon was investigated in *R. salmoninarum* challenge experiments during 1998 - 2000. To carry this out, test fish were infected by intraperitoneal injection of a virulent strain of *R. salmoninarum,* and after a nine-day incubation period, fed medicated rations. This final report describes the results obtained during 1998-1999 and 1999-2000 as described in the workplans submitted under this subtask.

Materials and Methods

Year 1998 – 1999

Approximately 2,000 Minter Creek fall chinook salmon smolts were transferred to a seawater tank at Manchester for the study. After acclimation to seawater 600 of these fish were challenged intraperitoneally with 1 x 10^6 colony forming units of *R. salmoninarum* strain ATCC 33209. Another 600 of these fish were injected intraperitoneally with an equivalent volume (0.1 ml) of phosphate buffered saline to duplicate the stress of handling and inoculation of the challenged fish. This group also served as a negative control for any deleterious effects of subsequent antibiotic treatment, activation of quiescent pre-existing BKD, or the appearance of other unrelated disease and mortality.

After inoculation, the fish were randomized and transferred into circular 1.6 m diameter tanks, with each tank containing 75 fish. Ten days post challenge (a time period when first mortalities from this challenge usually occur) fish were switched to a feed with antibiotic supplement, as follows:

- BioDiet + no medication
- BioDiet + erythromycin (dosage to equal 100 mg/kg/d for 28 d; standard INAD treatment protocol)
- BioDiet + azithromycin (10 mg/kg/d for 14 d)
- BioDiet + azithromycin (30 mg/kg/d for 14 d)

Fish health and survival were monitored daily for approximately five months. Necropsies were performed on all dead fish and kidney tissues were excised and assayed for BKD by Fluorescent Antibody Test (FAT) (Elliott and Barila 1987).

Year 1999 - 2000

Experimental design for the 1999 – 2000 study was similar to that of the previous year except fish were vaccinated against vibriosis by intraperitoneal injection of Alpharma Alpha Dip 2100 *Vibrio anguillarum-ordalli* bacterin 15 days before seawater entry. Also in this trial one treatment (10 mg/kg azithromycin) was eliminated enabling triplicate groups of *R. salmoninarum* challenged fish. Due to tank number limitations, unchallenged/unmedicated and unchallenged/erythromycin groups were carried out in duplicate only.

Approximately 2,000 George Adams fall chinook (vibrio vaccinated) salmon smolts were transferred to a large (2.2 m diameter) seawater tank at Manchester for the study. During acclimation to seawater, randomly selected fish were screened for the presence of BKD by FAT and ELISA, and by RT-PCR of blood samples (Rhodes et al. 1998). Prior year pre-screening showed that ~25% of this stock of chinook were infected with *R. salmoninarum*, although clinical signs of disease were absent. After acclimation to seawater 600 of these fish were challenged intraperitoneally with 1 x 10^6 colony forming units of *R. salmoninarum* strain ATCC 33209. Another 600 fish were injected intraperitoneally with an equivalent volume (0.1 ml) of phosphate buffered saline to duplicate the stress of handling and inoculation of the challenged fish. This group also served as a negative control for any deleterious effects of subsequent antibiotic treatment, activation of quiescent pre-existing BKD, or the appearance of other unrelated disease and mortality. After inoculation, the fish were randomized and transferred into tanks (1.6 m diameter), with each tank containing 100 fish.

Ten days post-challenge fish were switched to a feed with antibiotic supplement. Triplicate groups of *R. salmoninarum*-challenged fish were fed:

- BioDiet + no medication.
- BioDiet + erythromycin (dosage to equal 100 mg/kg/d for 28 d; standard INAD treatment protocol).
- BioDiet + azithromycin (30 mg/kg/d for 14 d).

Approximately two weeks after the experiment began, one fish was randomly sampled from each of the sixteen tanks for FAT and ELISA (Pascho and Mulcahy 1987) for determination of background levels of *R. salmoninarum*. Fish health and survival was monitored daily for approximately seven months. Necropsies were performed on all mortality and kidney tissues sampled for determination of *R. salmoninarum* presence and levels via FAT and ELISA During the experiment, all fish were treated one time with a 1:4000 formalin solution for 1 hour because of *Ichthyobodo* parasitism and two 10 day treatments with oxytetracycline at 75 mg/kg/d for cutaneous infections with filamentous bacteria. Also during the fifth month of this experiment all surviving fish were given a repeat treatment of the original BioDiet + macrolide feeds.

Results

Year 1998 - 1999

Mortality at five months in the challenged chinook was 38% in fish treated with azithromycin 30 mg/kg/d for 14 days (Figure 1). Challenged chinook treated with 10 mg/kg/d for 14 days had a cumulative mortality of 93%, and both fish that received 100 mg/kg/d erythromycin for 28 days and challenged fish fed the non-medicated ration had a death rate of 87% (Figure 1). Challenged fish treated with azithromycin at 10 mg/Kg/d for 14 d had final cumulative mortality approaching that of the non-treated challenged group (data not shown). In this IP-challenged group, approximately 67% of the mortality during the first 2.5 months of the experiment was due to BKD, as confirmed by clinical lesions and FAT. In the final 2.5 months, 77% of mortality was due to vibriosis and an unidentified filamentous bacterium. In the unchallenged groups that received the same treatments, mortality due to BKD was minimal (<10%) with the majority of losses due to vibriosis and tail and fin erosions caused by the unidentified filamentous bacterium.

Year 1999 - 2000

Three of the eight unchallenged chinook salmon randomly sampled two weeks after beginning the experiment and eight of eight *R. salmoninarum*-challenged fish had ELISA optical density (OD) levels over 0.250. These OD readings are suggestive of early development of BKD. Challenged fish treated with azithromycin had the least cumulative mortality (~15%) at termination of the experiment (Figure 2). However, survival of the unchallenged chinook fed erythromycin was only slightly less. Cumulative mortality in the remaining groups ranged from ~50% in the unchallenged azithromycin treated fish to ~ 65% in the unchallenged non-medicated controls (Figure 2). However, a statistical analysis (2-way Anova) of the cumulative mortality with all of the groups suggests that there are no significant differences in the survival of fish treated with either erythromycin or azithromycin (Table 1).

Three weeks after beginning the experiment, both challenged and unchallenged fish fish in all treatments began to die with severe cutaneous erosions that often resulted in complete ablation of the caudal fin. Wet mounts of lesion tissue revealed *Ichthyobodo* and a filamentous bacterium as possible causes. The parasite was successfully treated with formalin and the losses were controlled during the two 10 day treatments with oxytetracycline, but reoccurred after ending oral medication.

Discussion

Until effective vaccines or other treatments are developed to prevent or treat *R. salmoninarum* infections of salmon, antibiotics will continue to be used, particularly for captive broodstocks of endangered salmon stocks. In an earlier report it was demonstrated that the survival of sockeye salmon fed azithromycin (30 ppm) was significantly longer ($P < 0.001$) than the survival of sockeye salmon fed erythromycin (100 ppm) in long-term studies where the fish had been infected with *R. salmoninarum*

through cohabitation with diseased fish. In addition, sockeye salmon treated with azithromycin had a lower daily mean mortality rate (0.26%) than did sockeye on erythromycin (1.18%), or the non-medicated control feed (2.93%).

The results in these experiments have now been expanded to include chinook salmon, and also to include more rigorous controls and a more uniform method of challenging fish with *R. salmoninarum.* In order to mimic more closely how azithromycin would be used in a captive broodstock setting, an experimental protocol was devised where fish would be infected and then treated with azithromyin or erythromycin once disease symptoms became apparent. This was carried out by challenging fish intraperitoneally with a dose of *R. salmoninarum* known from prior experience in the laboratory to cause clinical BKD within three to four weeks. At the sign of first mortality, the fish were immediately switched to a diet containing erythromycin or azithromycin, as they would be in an outbreak of BKD in the captive broodstock program.

In the 1998 - 1999 experiment, onset of mortality in the challenged groups due to BKD was approximately 25 - 30 days following challenge. The challenged group that received no medication demonstrated a rapid rise in cumulative mortality that peaked at approximately 85% (Figure 1). In contrast, fish treated with erythromycin or azithromycin exhibited a much lower cumulative mortality (approximately 20%) 90 days after challenge. At this point, non BKD-related mortality compromised the erythromycin-treated fish. The same non BKD-related mortality affected the control (unchallenged fish) as well. While these latter results prevent rigorous statistical evaluation, the results observed with the challenged fish suggest that azithromycin is at least as efficacious as erythromycin in the treatment of BKD in chinook salmon.

A repeat of the study in the 1999 - 2000 time-frame included a vibrio vaccination in the hope that the non-BKD mortality experienced in the previous year could be avoided. Unfortunately, other pathogens appeared to cause mortality in all groups (*Ichthyobodo* and an non-speciated marine *Flexibacter* sp. bacterium). In this experiment it appears that the greatest survival rate in the challenged groups was again obtained with azithromycin (Figure 2). However, a detailed statistical analysis of the data obtained from all groups indicated no significant difference with either the challenge or the different drug treatments (Table 1).

Conclusion

Data collected to date suggests that the macrolide antibiotic azithromycin is more effective in reducing mortality due to BKD in captive broodstock salmon than erythromycin, the antibiotic approved for use against this disease. As azithromycin penetrates cells and has a longer half-life in tissue than erythromycin, this finding is not surprising. While this effectiveness was somewhat more pronounced in sockeye over chinook salmon in the studies carried out over the past several years, the measured differences in the studies presented here are not very large and it is proposed that the prescribed use of azithromycin be in only those cases where it is a last resort for

treatment of BKD outbreaks in endangered species. Yet to be determined is whether subclinical *R. salmoninarum* infections treated with azithromycin result in a complete curing of the organism.

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Table 1. Two-way Anova analysis of 1999-2000 antibiotic efficacy study comparing the survival of challenged (CH), unchallenged (UnCH), azithromycin (Az), and erithromycin (Em) treatments.

Days post-challenge	Comparison of:-	P value
60 days	CH versus UnCH	0.8540
	Az versus Em (UnCH)	0.5492
	Az versus Em (CH)	0.2523
165 days	CH versus UnCH	0.4930
	Az versus Em (UnCH)	0.2887
	Az versus Em (CH)	0.2261
240 days	CH versus UnCH	0.9337
	Az versus Em (UnCH)	0.2138
	Az versus Em (CH)	0.1021

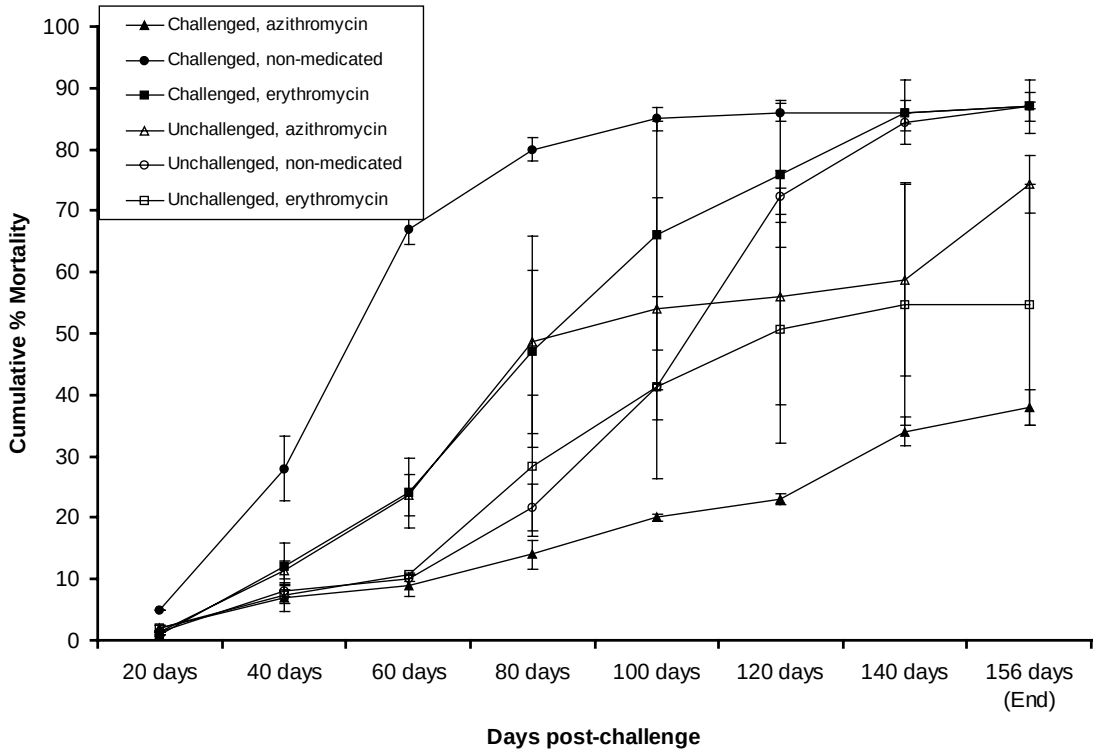

Figure 1. Cumulative % mortality in the 1998-1999 study of erythromycin and azithromycin efficacy against *R. salmoninarum* infections of chinook salmon. These experiments were carried out in duplicate (100 fish/tank, 2 tanks for each treatment). Error bars are $\pm$ 1 SE.

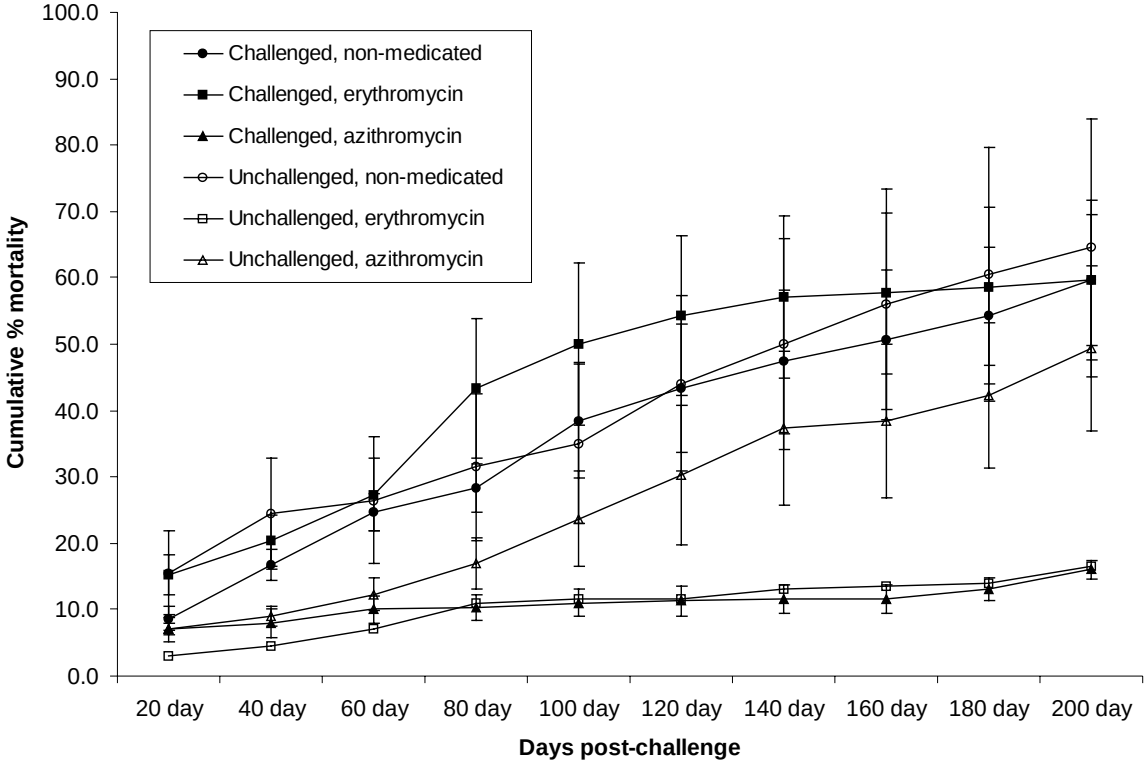

Figure 2. Cumulative % mortality in the 1999-2000 study of erythromycin and azithromycin efficacy against *R. salmoninarum* infections of chinook salmon. These experiments were carried out in triplicate (100 fish/tank, 3 tanks for each treatment). Error bars are $\pm$ 1 SE.